University of Alberta

Factors affecting cerebrovascular disease: hyperthermia and high-density lipoprotein

By

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Abstract

Hyperthermia worsens outcome of stroke in patients and also in animal models. In the present work, effects of hyperthermia on cerebral infarction and on the efficacy of thrombolytic therapy were examined in a rat model of embolic stroke. In Part A, efficacy of tissue plaminogen activator (tPA) treatment was examined in normothermic and hyperthermic rats. In Part B, brain perfusion deficits were assessed and in Part C, blood-brain barrier (BBB) permeability was examined. Finally, in Part D, the influence of hyperthermia on fibrinolytic activity of tPA was examined. Results showed that hyperthermia masks the neuroprotective effects of tPA since treatment with tPA significantly reduced infarct volume in normothermic and 38°C hyperthermic rats but not in 39°C rats. Perfusion deficits were also significantly increased in hyperthermic rats at both 3 h and 6 h after ischemic injury. Evans blue dye extravasation was significantly increased in the injured 39°C rats. *In vitro* study showed that hyperthermia increased the fibrinolytic activity of tPA.

Endothelial progenitor cells (EPCs) are implicated as an important marker of endothelial function and cardiovascular risk. In the present work, we examined whether high-density lipoprotein (HDL) cholesterol plays a role in the peripheral EPC levels and its underlying mechanisms in the HDL cholesterol-induced elevation of EPCs. For the clinical study, vascular risk factors and blood markers were measured and EPC colony forming units were counted after 7 days of culture. For the *in vitro* study, after 7 days of culture, EPCs were incubated in the presence or absence of HDL for 24 hours followed by measurements of endothelial nitric oxide (eNOS) and pro-matrix metalloproteinase-9

(pro-MMP-9) expression and caspase-3 activity. EPC colony levels significantly correlated with HDL levels. HDL treatment significantly increased eNOS protein expression in EPCs while it significantly decreased pro-MMP-9 levels at the concentration of 50μg/mL. Homocysteine treatment significantly increased caspase-3 activity whereas HDL significantly decreased it as compared to the homocysteine-only treated group.

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List of Abbreviations

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole
ANOVA = analysis of variance
apoA-I = apolipoprotein A-I
apoE = apolipoprotein E
ATP = adenosine triphosphate
AVM = arteriovenous malformations
BBB = blood-brain barrier
CAD = coronary artery disease
CBF = cerebral blood flow
CCA = common carotid artery
CNS = central nervous system
CPP = cerebral perfusion pressure
CSF = cerebral spinal fluid
CVR = cerebrovascular resistance
ECA = external carotid artery
eNOS = endothelial nitric oxide synthase
EPC = endothelial progenitor cells
FFA = free fatty acid
G-CSF = granulocyte-colony stimulating factor
GM-CSF = granulocyte monocyte-colony stimulating factor
H & E staining = hematoxylin and eosin staining
HDL = high-density lipoprotein
HMG-CoA = 3-hydroxy-3methylgluaryl coenzyme A
ICA = internal carotid artery
ICAM-1 = intercellular adhesion molecule-1
ICAM-1 = intercellular adhesion molecule-1
ICH = intracerebral hemorrhage
IL-1β = interleukin-1beta
IL-6 = interleukin-6
iNOS = inducible nitric oxide synthase
LDL = low-density lipoprotein
MAP2 = microtubule-associated protein 2
MCA = middle cerebral artery
MCAO = middle cerebral artery occlusion
MMP-9 = matrix metalloproteinase-9
MRI = magnetic resonance imaging
NINDS = National Institute of Neurological Disorders and Stroke
NMDA = N-methyl-D-aspartate
NO = nitric oxide
OCT = optimal cutting temperature
PE = polyethylene
PI3K/Akt = phosphatidylinositol 3-kinase/Akt
rICA = right internal carotid artery
rMCA = right middle cerebral artery
SAH = subarachnoid hemorrhage
SD = Sprague Dawley
SDF-1 = stromal cell derived factor-1
TIA = transient ischemic attack
TNF-α = tumor necrosis factor-alpha
tPA = tissue plasminogen activator
TTC = 2,3,5-triphenyltetrazolium chloride
VCAM-1 = vascular cell adhesion molecule-1

VEGF = vascular endothelial growth factor
VEGFR2 = vascular endothelial growth factor receptor 2

Chapter 1: Background

Cerebrovascular disease is the third leading cause of death in the United States, ranking behind cardiac disease and all forms of cancer (Demaerschalk 2003). Stroke is a leading cause of serious long-term disability in the West. According to American Stroke Association statistics, every 45 seconds someone in America has a stroke and every 3 minutes someone dies of one. Every year about 700,000 Americans experience a new or recurrent stroke, resulting in an estimated 163,538 stroke-related deaths. The economic burden of this disease is tremendous, costing the United States $40 billion to $50 billion per year, with a staggering $57.9 billion estimated for the 2006 fiscal year.

The numbers in Canada are not as high as those in the United States; however, they are high enough for the Heart and Stroke Foundation of Canada to designate stroke as the fourth leading cause of death in Canada. Every year, there are an estimated 40,000 to 50,000 stroke cases in Canada, resulting in approximately 16,000 deaths. As of 2002, about 300,000 Canadians are living with stroke. The loss of these patients from the work force and the extended hospitalization they require during recovery make the economic impact of this disease one of the most devastating in medicine. The impact of stroke on the Canadian economy is an estimated $2.7 billion per year, with Canadians spending a total of 3 million days in hospital because of stroke.

Besides testifying to th widespread prevalence of this devastating affliction, these statistics show that the currently available treatments for stroke are inadequate. Thrombolytic therapy with tissue plasminogen activator (tPA) is the only scientifically

proven effective therapy for acute ischemic stroke (Albers 1999; Traynelis and Lipton, 2001). However, strategies for appropriate management of stroke are complicated by the risk of cerebral hemorrhage associated with tPA use, as well as by the lack of information pertaining to its effectiveness when administered in the presence of any one of a number of adverse clinical profiles associated with stroke, such as hyperthermia or pyrexia. Clinical studies have shown that mild hyperthermia or fever in stroke patients can enlarge infarct size and worsen outcome of stroke; therefore, there exists a dire need to examine the efficacy of thrombolytic therapy under such adverse conditions. After a stroke has occurred, the focus of stroke management shifts from treatment to secondary stroke prevention which includes combating risk factors associated with stroke and maintenance of healthy vasculature. Therapies designed to improve vascular health can, therefore, greatly diminish the risk for developing future ischemic events.

Cerebrovascular disease

Cerebrovascular disease is caused by several pathophysiological processes involving the blood vessels. Atherosclerosis of the arteries, large and small, that supply the brain is the most common cause of cerebrovascular disease in North America and Europe. Other processes intrinsic to the vessel, such as lipohyalinosis, inflammation, amyloid deposition, arterial dissection, aneurismal dilation, venous thrombosis or development malformation, can also lead to cerebrovascular disease (Albers 2001; Kistler et al., 2004). Remotely originating factors, such as an embolus, from the heart or extracranial circulation, lodged in an intracranial vessel are also indicative of cerebrovascular disease. Inadequate cerebral blood flow due to decreased perfusion pressure or profound

hyperviscosity is another pathophysiological process that may be associated with cerebrovascular disease. Rupture of a blood vessel in the subarachnoid space or intracerebral tissue can result in cerebrovascular disease characterized by a hemorrhagic stroke whereas the other processes described above can lead to transient cerebral ischemia or ischemic stroke (Kistler et al., 2004).

Stroke: Categories and subtypes

Stroke is a heterogeneous syndrome caused by multiple disease mechanisms, but all result in a disruption of cerebral blood flow, leading to tissue damage (Markus 2004). Stroke can result from a blood clot blocking a vessel or an artery, or from rupture of a blood vessel, interrupting blood flow to an area of the brain. These two broad categories of stroke, hemorrhage and ischemia, are on opposite ends of the spectrum. Hemorrhage is characterized by too much blood within the cranial cavity, while ischemia is manifested in the form of too little blood to supply an adequate amount of oxygen and nutrients to a region of the brain. Blood clotting, although beneficial in everyday life, is dangerous in the case of stroke because clots can block arteries and cut off blood flow to a region of the brain, resulting in ischemia. The two categories of stroke can be further divided into subtypes with different causes, clinical presentations, outcomes, and treatment strategies. An ischemic stroke can present in two ways, embolic or thrombotic stroke, and there are also two types of hemorrhagic strokes resulting from rupture of an artery, namely intracerebral and subarachanoid hemorrhage (Caplan 2000).

Ischemic stroke

Blockade of an artery supplying the brain results in an ischemic stroke since the brain depends on its arteries to continually bring fresh oxygenated blood from the heart. If an artery is blocked, the neurons quickly run out of energy and become unable to carry out their normal functions. If the artery remains blocked for more than a few minutes, the neurons may die. Ischemic stroke can be caused by a number of diseases, but most commonly the problem is narrowing of arteries in the neck or head due to atherosclerosis. Blood cells can collect in these narrow areas and form blood clots. These blood clots block the artery where they are formed (thrombosis) or can dislodge and become trapped in arteries closer to the brain (embolism) (Caplan 1993).

Embolic stroke

In an embolic stroke, a blood clot (embolus) forms somewhere other than the brain (usually the heart) and travels through the bloodstream to the brain where it lodges itself in a blood vessel that is small enough to block its passage. The subsequent disruption of blood flow, to the area supplied by that vessel, results in immediate physical and neurological deficits. If the disruption is transient, the physical and neurological deficits resolve completely and the ischemic episode is referred to as a transient ischemic attack (TIA). However, if the blood flow is blocked for a longer period of time, the affected region of the brain undergoes irreversible tissue damage, resulting in an infarction, or tissue death (Caplan 1993; Kistler et al., 2004).

Thrombotic stroke

In a thrombotic stroke, diseased or damaged cerebral arteries become blocked by the formation of a blood clot within the brain. Clinically referred to as cerebral thrombosis or cerebral infarction, this type of event is responsible for almost 50% of all strokes. Thrombotic strokes often result from unhealthy blood vessels that are clogged with a buildup of fatty deposits and cholesterol. Atherosclerosis is by far the most common cause of local disease within the arteries that supply the brain. The atherosclerotic lesions within these arteries are often superimposed by white platelet-fibrin and red erythrocyte-fibrin thrombi. As the thrombi form on these artherosclerotic plaques, they block the artery where they form or they can dislodge or break up, giving rise to emboli that travel downstream and can precipitate an embolic stroke or a transient ischemic attack (TIA). Cerebral thrombosis can also be divided into an additional two categories that correlate with the location of the blockage within the brain: large vessel thrombosis and small vessel thrombosis (Kistler et al., 2004).

Large vessel thrombosis

Large vessel thrombosis occurs when the blockage is in one of the brain's larger blood-supplying arteries such as the carotid or middle cerebral artery. Large vessel thrombosis is the most common and best understood type of thrombotic stroke. A combination of long-term atherosclerosis followed by rapid blood clot formation is the major cause of large vessel thrombosis. Large artery atherosclerotic infarction occurs as a consequence of impediment to normal perfusion, usually caused by an occlusion due to atherosclerosis and coexisting thrombotic state (Albers 2001).

Small vessel thrombosis

Small vessel thrombosis is the term used to describe blockade of one (or more) of the brain's deeper penetrating arteries that supply deep structures such as the basal ganglia, internal capsule, and thalamus. Since these arteries penetrate deep into the cerebral structures, they are much smaller compared to the arteries on the surface of the brain and therefore stroke in these arteries is referred to as small vessel thrombosis or a lacunar stroke. The term lacunar is derived from the Latin word lacuna which means hole, and describes the deep cavities within the brain that result from death of the brain tissue supplied by the blocked small artery. The most common cause of obstruction of these arteries is lipohyalinosis, blockage by medial hypertrophy and lipid mixed with fibrinoid material in the hypertrophied arterial wall (Caplan 1989; Albers 2001).

Hemorrhagic stroke

Strokes caused by a sudden rupture of an artery are called hemorrhagic strokes. Hemorrhagic strokes can be caused by a number of disorders affecting the blood vessels, including high blood pressure and cerebral aneurysms. Aneurysms usually do not cause problems until they rupture and precipitate hemorrhagic strokes. Although hemorrhagic strokes account for only about 17% of all strokes, they have a much higher mortality rate. There are two subcategories of hemorrhagic stroke: intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). Although ICH and SAH are very similar, they generally result from different causes (Caplan 1992; Kase and Caplan 1996).

Intracerebral hemorrhage

Intracerebral hemorrhage (also called parenchymal hemorrhage) is defined as the rupturing of diseased cranial blood vessels, resulting in the leakage of blood into the surrounding brain tissue. The sudden increase in pressure within the brain can cause damage to the brain tissue surrounding the blood. A rapid increase in the amount of blood may also lead to unconsciousness or death due to sudden buildup of pressure. Intracerebral hemorrhage usually occurs in selected parts of the brain, including the basal ganglia, cerebellum, brainstem, or cortex. The most common risk factor for ICH is chronic hypertension; hypertension causes arteries and arterioles to become weakened, resulting in leakage. Excessive alcohol consumption and drug use, particularly of cocaine and amphetamines, are the most common causes of ICH for people in their 20s and 30s. Other less common causes of ICH include trauma, infections, tumors, blood clotting deficiencies, and blood vessel abnormalities such as arteriovenous malformations (AVM). In AVM, blood passes directly from an artery to a vein, instead of first passing through a capillary network. The resultant high pressure in the draining veins may cause them to rupture and bleed (Caplan 1992; Kase and Caplan 1996).

Subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) results from bleeding into the skull or cranium that occurs when a blood vessel on the surface of the brain ruptures and bleeds into the meningeal subarachnoid space. The blood spreads quickly within the cerebral spinal fluid (CSF), rapidly increasing intracranial pressure. The sudden buildup of pressure can result in rapid loss of consciousness or death. Symptoms of SAH begin abruptly, in

contrast to the more gradual onset of ICH. SAH is most often caused by rupture of arterial aneurysms that lie at the base of the brain or from vascular malformations that lie near the pial surface (Gorelick et al., 1986; Linn et al., 1994).

Cerebral perfusion

In order to manage high metabolic demands of the brain and the devastating consequences of cerebral ischemia, the cerebral circulation has developed several specialized features. For a relative size of only 2%, the brain oxygen requirement in the adult human accounts for about 20% of that of the whole body. This high metabolic demand is matched by a disproportionately large amount of cardiac output to the brain. In a healthy individual, total cerebral blood flow is about 800 mL/min or approximately 15-20% of total cardiac output. Cerebral perfusion is characterized by a high flow, low pressure system with relatively preserved diastolic flow. As opposed to the systemic circulation, the ratio of diastolic to systolic phase blood flow is much higher in cerebral circulation. Natural variations in cerebrovascular anatomy play a major role in determining whether an occlusion results in a massive hemispheric stroke or is asymptomatic (Markus 2004).

Cerebral circulation has a well developed collateral circulation, particularly the circle of Willis at the base of the brain, which plays an important and protective role. Anastomoses between the external carotid artery branches and the intracerebral circulation may also serve as collateral supply (Riggs and Rupp 1963). Despite this collateral supply, certain regions in the brain are still particularly vulnerable to an

inadequate cerebral perfusion. Territories supplied by the perforating arteries that supply the white matter and subcortical grey matter nuclei are particularly vulnerable to ischemia as are the regions at the boundaries between the anterior and middle and the middle and posterior cerebral arteries. The penetrating or perforating arteries that supply the deep structures of the brain are end arteries and therefore ischemia in their territory results in more rapid cell death (Schmidt et al., 1991, Markus 2004).

Cerebral circulation and response to focal ischemia

Under normal conditions cerebral perfusion pressure (CPP) and cerebrovascular resistance (CVR) are the major determinants of cerebral blood flow (CBF). CPP is due to the difference between systemic arterial pressure and venous back pressure and CVR is determined by the diameter of the intracranial arteries and also influenced by blood viscosity. Healthy brain tissue receives a steady blood supply averaging 50 mL per 100g of brain tissue per minute (Schmidt et al., 1991). In pathological conditions, such as blockage or severe stenosis of an intracranial supplying artery, as CPP falls, intracranial resistance vessels dilate to maintain CBF, resulting in an increased cerebral blood volume. Cerebral artery occlusion results in a decrease in perfusion pressure (Kishi et al., 2000), pH (Kajita et al., 1998), and blood oxygen content (Brown et al., 1985) and an increase in carbon dioxide (Morita et al., 1994) content downstream from the occlusion. All of these factors contribute to vasodilation. Further falls in CPP cannot be compensated for when vasodilatation is maximal, and this results in a fall in CBF. Metabolic activity can still be maintained initially by increasing the oxygen extraction fraction (OEF) from blood because normally oxygen delivery to the brain greatly exceeds

demand (Frizzell et al., 1991). When no further increases in oxygen extraction can occur and the flow is inadequate to meet metabolic demands, cellular metabolism is impaired, leading to cellular, biochemical, and metabolic consequences of cerebral ischemia.

Experimental studies in baboons and clinical studies in humans have shown that the spontaneous or evoked electrical activity is compromised or ceases when CBF falls below 16 – 18 mL/100g/min. This represents a threshold for loss of neuronal electrical function, resulting in a loss of ability of neurons to fire action potentials, and thus neurological deficits appear (Jones et al., 1981). Adenosine triphosphate (ATP) content may be normal or only slightly below normal and the physiological membrane potential is still intact at this point (Naritomi et al., 1988). In addition, extracellular levels of excitatory amino acids (glutamate and aspartate) begin to rise (Shimada et al., 1989). A lower threshold of 10 – 12 mL/100g/min exists for loss of cellular ion homeostasis that maintains membrane function. At this point, glycolysis ceases and ATP levels diminish (Swanson et al., 1997). At this lower threshold, K^+ is released from and Ca^{2+} is taken up by the cells (Harris et al., 1981). Rapid efflux of K^+ and uptake of Ca^{2+} represents a generalized collapse of membrane function. Membrane gradients cannot be maintained and cells also take up Na^+ and Cl^-; water follows passively, resulting in edema which can affect the perfusion of the area surrounding the ischemic core by increasing intracranial pressure and vascular compression (Siesjo 1992). Increased K^+ efflux and Ca^{2+} influx cause ischemic neurons and glia to undergo anoxic depolarization in the ischemic core from which the cells cannot repolarize. In penumbral regions, however, cells may repolarize but at the expense of further energy consumption, causing lethal waves of

spreading depolarization that contribute to excitotoxicity (Nielson et al., 2000; Barber et al., 2001). As the number of peri-infarct depolarizations increase, so does the infarct size by increasing excitotoxicity.

Excitotoxicity is primarily due to Ca^{2+} overload. Glutamate release by depolarized ischemic neurons results in increased calcium and sodium influx into the cells through activation of postsynaptic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptors. Energy-dependent processes such as glutamate re-uptake are impaired, leading to further increases in extracellular glutamate levels and increased Ca^{2+} influx (Siesjo et al., 1989). This, combined with the inability of neurons to extrude intracellular Ca^{2+} ions (lack of ATP), allows Ca^{2+} levels to reach toxic levels within neurons (Choi and Rotham 1990; Katsura et al., 1994). The increased calcium and sodium influx into the cells leads to cellular edema and activation of the catabolic processes that destroy cellular integrity. The threshold for infarction appears to be similar to that for energy failure/loss of membrane homeostasis, but it varies with the duration of the insult (Mody and MacDonald 1995; Choi et al., 1998). The disruption of calcium homeostasis leads to activation of a series of Ca^{2+}-dependent processes, such as activation of proteolytic enzymes that degrade both the cytoskeletal and extracellular matrix proteins (Zhao et al., 1994).

Normally, intracellular Ca^{2+} levels are tightly controlled because Ca^{2+} is involved in the activation of numerous enzymes which regulate a multitude of biochemical pathways. Increases in Ca^{2+} concentrations that take place during normal conditions, such as

neurotransmitter release, are very brief in time and occur as localized gradients within the cytoplasmic space. The cytotoxic increase in Ca^{2+} observed in ischemic neurons results in the inappropriate activation of Ca^{2+}-dependent processes which contribute to cell death via necrosis or apoptosis. Ca^{2+} activates proteolytic enzymes such as calpain. Calpain is a calcium-sensitive cysteine protease that when activated, degrades neuronal cytoskeletal proteins such as spectrin and microtubule-associated protein 2 (MAP2). Calpain activation and spectrin proteolysis have been implicated in neuronal injury produced by hypoxia and ischemia (Ginsberg and Busto 1998; Minger et al., 1998). They also activate caspases (Blomgren et al., 2001), which stimulate apoptosis (Seshagiri and Miller, 1997).

Lipolytic enzymes like Ca^{2+}-dependent phospholipase A_2 are also activated by the high intracellular Ca^{2+} levels in ischemic neurons. Free fatty acids (FFAs) are elevated in the brain following both ischemic and traumatic injury. Phospholipases degrade the phospholipid cell membrane and increase the intracellular FFA concentrations by up to 20 times (Gardiner et al., 1981). Phospholipase activity can be regulated by calcium, by phosphorylation, and by agonists binding to G-protein-coupled receptors. These enzymes normally function in the physiological remodeling of cellular membranes, whereby FFAs are removed from the membrane by phospholipase activity and then reacylated with a different FFA. However, reductions in the cell's ability to maintain normal metabolic function and the resultant fall in ATP levels can cause the failure of reacylation of membrane phospholipids. Alterations to membrane phospholipids would be expected to compromise many cellular functions, including the ability to accumulate excitotoxic

amino acids. Degradation of membrane phospholipids as well as membrane instability due to rising fatty acid levels contributes to disintegration of the cell membranes. There is a correlation between the elevation of FFAs in the CSF and worsened outcome following stroke, traumatic brain injury, and subarachnoid hemorrhage in humans. The released FFAs, especially arachidonic and docosahexaenoic acids, together with the production of lysophospholipids, can initiate a chain of events which may be responsible for the development of neuronal damage. Inhibitors of both cyclooxygenase and lipoxygenase pathways have been shown to reduce cerebral deficits following ischemia and trauma (Phillis and O'Regan 2003).

Arachidonic acid is the primary FFA released by lipolysis during ischemia (Gardiner et al., 1991). With reperfusion and reestablishment of oxygen supply, arachidonic acid becomes a substrate for the cyclo-oxygenase and lipoxygenase pathways (Wieloch and Siesjo, 1982). Arachidonic acid metabolism results in formation of reactive oxygen species, lipid peroxides, and toxic aldehydes (malondialdehyde, 4-hydroxynonenal, and acrolein). Reactive oxygen species have been implicated in brain injury after cerebral ischemia. These oxidants can damage proteins, lipids, and DNA, and lead to cell injury and necrosis. Oxidants are also initiators in intracellular cell death signaling pathways that may lead to apoptosis. The possible targets of this redox signaling include mitochondria, death membrane receptors, and DNA repair enzymes (Kondo et al., 1997; Sugawara and Chan 2003). Mitochondria have been increasingly implicated in the pathophysiology of ischemic brain injury. Reactive oxygen species from arachidonic acid metabolism impair mitochondrial function by disrupting the inner mitochondrial

membrane and oxidizing proteins that mediate electron transport. Mitochondrial membranes become leaky and mitochondria become overloaded with Ca^{2+}, resulting in impaired ATP production (Budd 1998). Leaky mitochondria also release cytochrome C which acts as a trigger for apoptosis (Sugawara et al., 1999).

The lipoxygenase enzymes catalyze metabolism of arachidonic acid into leukotrienes (Ohtsuki et al., 1995). Leukotriene disruption of the BBB leads to formation of vasogenic edema (Mabe et al., 1990; Cipolla et al., 2004). Leukotrienes serve as chemoattractants to leukocytes, stimulating their adhesion to the vascular endothelium and migration through the BBB into the neuronal tissue, resulting in disruption of the BBB (Aktan et al., 1992). Ischemia is also correlated with microglial activation which releases inflammatory cytokines, many of which are responsible for neuronal damage (Saito et al., 1996; Flavin and Ho 1999). Inflammation is very important in extending ischemic injury. Proinflammatory cytokines have been implicated in several mechanisms that may potentiate ischemic brain injury, including production of nitric oxide (NO) from inducible nitric oxide synthase (iNOS) by astrocytes, recruitment, activation, and adhesion of infiltrating leucocytes to the endothelium, promotion of a local procoagulant state, and the regulation of apoptotic processes (Vila et al., 2000).

Apoptosis in acute stroke

Apoptosis is critical for development and maintenance of healthy tissues. There is much evidence to support the active participation of apoptotic processes in progressive neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, as well as in

acute ischemic stroke (Choi 1996). During focal ischemia, CBF drops below 10% of control values in the area proximal to the occlusion site, the infarct core. As a consequence of this, ATP levels decrease dramatically. The rapidity of pan-cellular death and histochemical reactivity have led to the idea that cell death in the infarct core is necrotic and therefore out of therapeutic reach. In the penumbral region surrounding the infarct core, CBF levels are maintained at about 40% of control values due to retrograde perfusion by anastomoses from adjacent arteries. Availability of limited amounts of oxygen and nutrients allows ATP levels to remain high enough to allow for the energy-dependent mechanism of apoptosis (Guegan and Sola 2000). However, this classification of cell death types in the core and the penumbral region is controversial as it is reported that ultrastructural observations of dying neurons in the core did not correlate with the original descriptions of necrosis. During development, necrosis is characterized by cell swelling, and rupture of protoplasmic and nuclear membranes, which results in a strong inflammatory reaction due to the spilling of cell content into the extracellular space. However, in the infarct core, apoptotic characteristics such as cytoplasmic and nuclear condensation with preserved membranes are seen more frequently than necrotic features (Wyllie et al., 1980). Protoplasmic astrocytes are the only cells displaying clear necrotic features after distal occlusion of the MCA (Lukaszevicz et al., 2002). Neurons never show extensive swelling, except for swelling of the endoplasmic reticulum, which is one of the first signs of apoptosis failure (Onteniente et al., 2003). In addition, neurons of the core also contain a number of activated caspases, suggesting that apoptosis is also involved in the initial phase of cerebral infarction (Velier et al., 1999; Benchoua et al., 2001). Caspases were initially thought to participate in the secondary step of the

infarction process, resulting in an expansion of the focal ischemic lesion into the penumbral region. Recent findings show that several caspases, including caspase-1 and -8, are strongly activated in the core region within a half-hour following permanent middle cerebral artery occlusion (MCAO). At this time, neurons of the penumbra have no structural abnormalities but some neurons in the core display a number of morphological features of early apoptosis such as cytoplasmic condensation or dilatation of the endoplasmic reticulum. This indicates that apoptosis is actually triggered in the core, and may well be the default mechanism of cell death following acute ischemia (Benchoua et al., 2002; Nicotera et al., 2000). Cell death in the core and in the penumbra involves distinct cascades of caspase activation, depending on the available energy levels. Neuronal degeneration in the core is mediated by activation of energy-independent pathways that include the "death-receptors" and the caspase-1 pathways. In contrast, the energy dependent apoptosome of the mitochondrial pathway is involved in the secondary expansion of the lesion into the penumbral region (Benchoua et al., 2001; Cecconi 1999)

Ischemic penumbra

The region of brain tissue surrounding the ischemic core that receives moderate blood flow (more than 15 mL/100g/min) is termed the penumbra. The concept of ischemic penumbra is crucial to the treatment of acute stroke. Following a focal ischemic insult, there exists a penumbral region around a core of densely ischemic and irreversibly damaged tissue. These cells contribute to neurological deficit as they lose their electrical function but do not show the release of potassium that characterizes cell death. These functionally silent cells are nevertheless viable as they regain their function and do not

undergo necrosis if flow is restored early enough (Markus 2004). Thus a window of opportunity exists to salvage this tissue, and the treatments for ischemic stroke center around preventing the penumbra from degenerating to infarction. Studies have shown that the duration of ischemia, as well as the absolute cerebral blood flow, plays a critical role in determining the fate of ischemic penumbra (Jones et al., 1981; Kaplan et al., 1991; Heiss et al., 1992). Ischemic tissue in the macaque monkey can withstand about three hours of occlusion when the CBF is around 15 mL/100g/min; however, if the CBF drops to 5 mL/100g/min, the same tissue can only withstand two hours of occlusion (Jones et al., 1981). This time dependence for transition from reversible to irreversible injury has crucial significance as it implies that acute treatment for stroke will be most successful when it is given as early as possible. Experimental data have demonstrated a gradual progression of reversible ischemia to irreversible infarction involving a complex cascade of mechanisms including blood flow and cellular and metabolic consequences of hypoperfusion (Jones et al., 1981; DeGirolami et al., 1984; Weinstein et al., 1986).

Hyperthermia in cerebral ischemia

Hyperthermia is characterized by an increase in temperature to levels higher than the body's thermoregulatory set-point of 37°C. An increase in core body temperature is an adaptive response to pathogens since the increased temperature will speed up the actions of the immune system and may also directly neutralize the pathogens. Hyperthermia following ischemic stroke is a common but undesirable event; however, its pathophysiology and clinical importance are still not fully recognized. Recently, there has been increasing evidence that moderate hyperthermia, when present during or after a

period of brain ischemia or trauma, markedly exacerbates the degree of resulting neural injury (Ginsberg and Busto 1998). Hyperthermia following ischemic stroke may result as a consequence of the brain infarct itself; however, the progress of biochemical and inflammatory responses associated with cerebral ischemia may also play an important role (Zaremba 2004). A spontaneous increase in body temperature has been observed following cerebral ischemia (Kato et al., 1990; Kuluz et al., 1993). As ischemia progresses and reperfusion of the affected tissue occurs, a number of proinflammatory genes are upregulated, promoting the development of an inflammatory reaction, which contributes to accentuation of cerebral injury (Castillo and Leira 2001; Emsley and Tyrrell 2002). The proinflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and interleukin-6 (IL-6) mediate most of the inflammatory events associated with cerebral ischemia. These cytokines promote leukocyte recruitment and trafficking into the ischemic brain through leukocyte activation, upregulation of complementary adhesion molecules on cerebral microvessels and circulating leukocytes, and chemokine release (Becker 1998). Leukocytes release a number of potentially harmful substances, including free oxygen radicals and proinflammatory cytokines, exerting a detrimental effect on ischemic brain. Apart from promoting leukocyte influx, inflammation is also typically characterized by hyperthermia (Becker 2001). These proinflammatory cytokines, TNF-α, IL-1β, and IL-6, which are pivotal for the inflammatory response, are known to be pyrogenic (Luheshi et al., 1996, Luheshi et al., 1997). In acute ischemic stroke patients, a highly positive correlation was seen between increased body temperature and increased plasma IL-6 levels (Vila et al., 2000). This suggests that hyperthermia following ischemic stroke may be a result of a

stroke-induced inflammatory reaction. However, the size of brain infarct also plays a role in determining the development of clinically measurable early poststroke hyperthermia. Increased body temperature was positively correlated with the initial stroke severity in that the increased body temperature within hours after stroke onset was related to major, but not mild or moderate strokes (Reith et al., 1996; Boysen and Christensen 2001). This may explain why not all ischemic strokes are accompanied by hyperthermia, even though the inflammatory response appears in virtually all ischemic strokes (Castillo and Leira 2001).

Hyperthermia during cerebral ischemia in animal studies

The effect of temperature alterations on cerebral ischemia has been extensively studied in laboratory animals, indicating that hyperthermia results in both exacerbation of ischemic brain injury and worsening of stroke outcome.

An increase in body temperature to 40°C twenty-four hours after focal cerebral ischemia resulted in a 3-fold increase in cerebral infarct volume (Kim et al., 1996). In standardized animal models of transient forebrain ischemia, intra-ischemic brain temperature elevations to 39°C were reported to enhance and accelerate ischemic injury in vulnerable brain regions and induce damage to structures not ordinarily affected (Dietrich et al., 1990a). Hyperthermia accelerated the appearance of severe neuropathological alterations in the hippocampus and striatum and led to marked damage to the superficial layers of neocortex, and to the infracted areas in cortex, thalamus, cerebellum, and substantia nigra (Busto et al., 1987; Dietrich et al., 1990a). Hyperthermia also adversely affected

behavioral consequences and survival in ischemic animals. In one study, hyperthermic rats tended to remain unresponsive to various stimuli and to die soon after ischemia (Dietrich et al., 1990a). In dogs subjected to 12.5 minutes of complete cerebral ischemia, those animals maintained at 37°C were behaviorally normal or near normal, while dogs maintained at 39°C either remained comatose or died, and animals held at 38°C had an intermediate outcome (Wass et al., 1995). In a model of reversible proximal middle cerebral artery (MCA) occlusion in rats, a 3-fold increase in infarction volume, as measured histologically, was reported with the elevation of brain temperature from 36°C to 39°C during a 2-hour period of MCA occlusion (Morikawa et al., 1992). A permanent model of MCA occlusion produced a 1.5- to 1.6-fold increase in infarction volume with hyperthermia (40°C) for one hour or more following ischemic injury (Chen et al., 1991).

Other studies in focal cerebral ischemia have shown that hyperthermia (39°C) is deleterious in focal ischemia and that the effects of otherwise neuroprotective drugs in ischemia may be nullified or become deleterious due to hyperthermia (Memezawa et al., 1995; Ginsberg and Busto 1998). MK-801, an N-methyl-D-aspartate antagonist known to be protective in focal ischemia, markedly reduced infarction volume in normothermic rats but failed to have a therapeutic effect in hyperthermic (39°C to 39.5°C) animals (Memezawa et al., 1995).

Hyperthermia during cerebral ischemia in human studies

Studies investigating the effects of hyperthermia during cerebral ischemia in humans have shown a relationship between increased body temperature in stroke patients and greater brain infarct size or poorer outcome. There is a linear relationship between the body temperature of stroke patients upon admission to emergency wards and the resultant severity of outcome of these patients over time (Reith et al., 1996; Wang et al., 2000). Clinical studies have indicated that 50% of all stroke patients have escalated body temperatures, as measured systemically, within 48 h of a stroke insult (Azzimondi et al., 1995; Kammersgaard et al., 2002). One study, examining the effects of hyperthermia at different times within the first 72 hours from the beginning of stroke symptoms showed that only hyperthermia appearing within the first 24 hours after stroke was independently related to larger brain infarct size and increased stroke severity. Hyperthermia within 72 hours was a predictor of poorer outcome with significantly increased poststroke mortality (Castillo et al., 1998). Hyperthermia in patients within the first 24 hours after stroke onset has also been shown to be associated with significantly higher stroke severity measured at discharge or at 6 months after onset (Reith et al., 1996). In a prospective study of 390 cases of acute stroke, admission body temperature proved to be highly correlated with initial stroke severity, infarct size, mortality, and poor outcome. For a 1°C difference in body temperature upon admission, the relative risk of poor outcome increased by 2.2-fold (Reith et al., 1996). A large meta-analysis of nine clinical studies, with a total of 3790 patients, on the effects of post-stroke pyrexia on stroke outcome found that pyrexia after stroke is associated with marked increase in morbidity and

mortality; therefore, measures should be taken to combat fever in the clinical setting to prevent stroke progression (Hajat et al., 2000).

Hyperthermia-mediated worsening of cerebral ischemia

Hyperthermia may act through several mechanisms to worsen cerebral ischemia. There are numerous reports indicating that temperature affects mechanisms of cerebral ischemia and influences neuronal survival in the ischemic penumbra (Chopp et al., 1988; Ginsberg and Busto 1998). The list of temperature-sensitive mechanisms that could modulate the outcome of cerebral ischemia is very long and can include mechanisms such as changes in cerebral blood flow, central nervous system (CNS) metabolism, neurotransmitter levels, edema formation, glucose metabolism, platelet function, FFAs, growth factors, BBB, calcium-dependent protein phosphorylation, protein kinase-C activity, protein synthesis, leukocyte accumulation, NMDA-induced neurotoxicity, heat shock proteins, cytoskeletal proteins, pH, cytokines, calcium accumulation, lipid peroxidation, and changes in free radical activation (Dietrich 1999).

Another important deleterious factor arising from increased core temperatures is the escalation of metabolic rate. The metabolic rate increases by 13% for every degree Celsius rise in core temperature above 37.5°C (Holtzclaw 1992). In the ischemic penumbra, cellular metabolism is maintained at a level sufficient to sustain essential neuronal functions. Further progression of cerebral ischemia within the penumbra causes its conversion into an irreversible lesion (Castillo and Leira 2001). The increased metabolic demand is especially harmful to these ischemic neurons, as their available

blood supply is not adequate enough to meet the increased demand for energy. The increase in metabolic rate and decrease in blood supply therefore creates an imbalance between energy supply and demand after cerebral ischemia. The magnitude of this imbalance is decreased by hypothermia, which decreases the metabolic rate of the brain, and is increased by hyperthermia, as the metabolic rate of the brain increases with the rise in temperature (Wood and Gonzales 1996). Therefore, in a hyperthermic ischemic brain, ATP and creatine phosphate levels will fall quickly, causing a greater rate of cell death than in normothermic ischemic brains (Thornhill and Corbett 2001).

Experimental studies in animal models of cerebral ischemia have shown that temperature-mediated changes in the metabolic rate of the ischemic brain may contribute to the neuroprotective effect of hypothermia and to the neurotoxic effect of hyperthermia on the ischemic brain (Ginsberg et al., 1992; Maher and Hachinski 1993). Hypothermia delays ATP depletion in ischemic neurons and also decreases the ischemia-induced accumulation of lactate (Chopp et al., 1988; Ginsberg et al., 1992), whereas lactic acidosis is increased by hyperthermia, resulting in acceleration of ischemic death (Ginsberg and Busto 1998; Maher and Hachinski 1993).

In one study, in cats subjected to 16 minutes of global cerebral ischemia, hyperthermia induced 1 hour or more before ischemia and maintained for 1.5 to 2 hours after recirculation exacerbated metabolic compromise in the experimental animals. Hyperthermia enhanced the degree of intracellular acidosis and impaired the recovery of cerebral ATP and phosphocreatine levels compared with normothermic cats (Chopp et

al., 1988). In another study, regional brain energy metabolites were assayed in rats recovering from 20 minutes of high-grade global ischemia performed at different cranial temperatures. The authors reported deficits in recovery of ATP levels and adenylate energy charge in both cortex and subcortical regions of rats with intraischemic hyperthermia of 39°C (Ginsberg et al., 1992).

Animal models of stroke have also provided evidence for temperature-dependent changes in intraneuronal calcium accumulation (Dietrich et al., 1996) and in the levels of the various protein kinases influenced by ischemia (Busto et al., 1994). More specifically, hyperthermia (39°C) during ischemia was found to exacerbate the degree of inhibition of calcium/calmodulin-dependent protein kinase II induced by a brief period of global ischemia. Calcium/calmodulin-dependent protein kinase II is a mediator of many of the second-messenger effects of calcium, including neuronal excitability, synaptic transmission and modulation, cytoskeletal function, and the release of such neuroexcitatory amino acids as glutamate and glycine (Churn et al., 1990). In another study, hyperthermia was also reported to significantly influence the patterns of protein kinase C alteration induced by global ischemia (Busto et al., 1994).

Hyperthermia, during both global and focal ischemia, has also been shown to significantly alter the release of neurotransmitters. Elevations in glutamate and glycine levels after ischemic brain injury have been shown to be accentuated by hyperthermia (Ginsberg and Busto 1998) and attenuated by hypothermia (Baker et al., 1991). These changes in neurotransmitter release may contribute to the hyperthermia-dependent

evolution of ischemic brain damage (Dietrich et al., 1996). A microdialysis study examining the effects of hyperthermia mediated changes in neurotransmitter release in rats undergoing 20-minute forebrain global ischemia showed a 21-fold increase in basal ganglionic glutamate levels during normothermic ischemia, which returned to normal by 20 to 30 minutes of circulation. By contrast, glutamate levels in hyperthermic (39°C) rats were increased by 37-fold and persisted for a longer period of time during the recirculation period (Sternau et al., 1992). In addition to this, the excitotoxic index, a composite measure of neurotransmitter release, was shown to increase by only 2-fold in normothermic rats but showed a 20-fold elevation in hyperthermic ischemic rats, suggesting a potential for greatly enhanced excitotoxicity under hyperthermic conditions (Globus et al., 1991; Sternau et al., 1992). In a focal ischemia study, using 2-hour temporary MCA clip-occlusion in rats, hyperthermia (39°C) during MCA occlusion increased the average peak glutamate release in the penumbral cortex by 31-fold above baseline compared with only 6.5-fold elevation in normothermic (37°C) rats (Takagi et al., 1994). Increases in glutamate levels with hyperthermia have also been reported in clinical stroke settings. One study reported a glutamate-associated relation between an increase in body temperature and the worsening of symptoms as well as enlargement of brain infarction volume in ischemic stroke patients (Castillo et al., 1999). The authors examined a mechanism for hyperthermia-mediated aggravation of cerebral injury in acute ischemia by studying the relationship between body temperature on admission and CSF concentrations of neuroexcitatory amino acids in 128 patients with acute ischemic stroke of less than 24 h duration. Glutamate concentration was significantly increased in hyperthermic (body temperature >37.5°C) as compared to normothermic patients. The

same study also revealed the glycine concentration to be significantly increased in hyperthermic patients as compared to normothermic. The authors concluded that glutamate and glycine release during the acute phase of cerebral ischemia could be responsible for the increased neuronal damage seen with hyperthermia (Castillo et al., 1999), providing direct clinical evidence for hyperthermia-mediated changes in neurotransmitter levels resulting in worsening of ischemic injury.

Another mechanism associated with hyperthermia-mediated worsening of cerebral ischemia is the change in oxygen radical production. Hyperthermia has been shown to increase cortical oxygen radical production during the early recirculation period after a global ischemia insult in rats (Globus et al., 1995). This study used *in vivo* microdialysis to analyze the brain's extracellular fluid for evidence of hydroxyl radical production by measuring formation of 2,3- and 2,5-dihydroxybenzoic acid (a measure of free radical formation). The authors reported that cortical oxygen radical production during the early recirculation period after a global ischemic insult is greatly influenced by intraischemic brain temperature. Hyperthermia in the setting of ischemia resulted in a 2-3 fold increase in 2,5-dihydrobenzoic acid compared with normothermic conditions (Globus et al., 1995).

In relation to stroke-induced inflammatory reaction, hyperthermia is implicated in maximal activation of leukocytes, promoting the cytotoxic function of inflammatory cells. Hyperthermia may also influence dysfunction of the BBB, facilitating the influx of leukocytes following the stroke-induced inflammatory reaction. Ischemia-induced BBB

opening is remarkably sensitive to brain temperature. Moderate intraischemic hypothermia of 30°C to 33°C has been shown to attenuate the mild extravastion of protein tracers across the BBB observed after periods of normothermic ischemia, whereas intraischemic hyperthermia (39°C) markedly exaggerated this extravasation of protein tracers (Dietrich et al., 1990b). The temperature-sensitive nature of the BBB could be responsible for the progression of inflammatory related ischemic brain damage due to hyperthermia. This is due to the increased permeability of the BBB associated with increased transfer of leukocyte recruitment mediators across the barrier following hyperthermia in ischemic rats (Dietrich et al., 1991).

Temperature changes may also influence the expression of adhesion molecules following stroke-induced inflammatory reaction, as hyperthermia increases intercellular adhesion molecule-1 (ICAM-1) expression and lymphocyte adhesion to endothelial cells (Lefor et al., 1994) whereas hypothermia significantly reduces the upregulation of ICAM-1 mRNA and the accumulation of leukocytes after focal cerebral ischemia in rats (Kawai et al., 2000). Hyperthermia is also implicated in increased cytoskeletal damage following cerebral ischemia. Activation of calpain, a calcium-sensitive cysteine protease, and spectrin proteolysis is seen in neuronal injury produced by hypoxia and ischemia. Calpain is involved in degradation of neuronal cytoskeletal proteins such as spectrin and microtubule-associated protein 2 (MAP2). In a study examining the effects of hyperthermia on spectrin proteolysis, hyperthermia (39°C), during 1-hour transient proximal MCA occlusion, led to spectrin proteolysis in cortical pyramidal neurons soon after onset of reperfusion, which, in association with morphological evidence of

irreversible neuronal injury became marked by 4 and 24 hours. However, in the normothermic animals only a few neurons showed spectrin proteolysis at reperfusion and this effect subsided by 24 hours (Morimoto et al., 1997). In a related study, intraischemic hyperthermia was reported to significantly decrease calmodulin and MAP2 immunoreactivity in the vulnerable hippocampal CA1 neurons of gerbils undergoing 5 minutes of global forebrain ischemia (Eguchi et al., 1997).

In summary, hyperthermia is an important event accentuating biochemical and inflammatory ischemic mechanisms within the ischemic penumbra, thus contributing to worsening of outcome of cerebral ischemia and, therefore, should be combated assiduously in stroke patients.

Therapeutic implications in light of hyperthermia during stroke

Due to the limited knowledge of the mechanisms of poststroke hyperthermia, no specific treatment for this condition has been developed. Antipyretic treatments, such as aspirin, have shown no efficacy in experimental animals (Legos et al., 2002). Nevertheless, because of its antiplatelet actions, aspirin is recommended in ischemic strokes, provided there are no other contraindications (Zaremba 2004). Clinical trials have also recently examined the possibility of physical or chemical cooling therapy in acute stroke, assuming that induced hypothermia might result in improved stroke outcome. One study reported that treatment of acute stroke patients with induced hypothermia down to 32±1°C for 12 to 72 hours appears feasible and safe but requires refinements of the cooling process, duration of therapy, and most importantly evaluation of clinical efficacy

(Krieger et al., 2001). In another study, moderate hypothermia (33°C) for 48 to 72 hours in 25 patients with MCA stroke and elevated intracranial pressure significantly reduced intracranial pressure and suggested improved long-term outcome among survivors (Schwab et al., 1998). Evidence from pharmacological inducers of hypothermia is not very convincing. A randomized and controlled clinical trial of early administration of acetaminophen (paracetamol) to afebrile acute stroke patients failed to show significant effects on hypothermia promotion, hyperthermia prevention, and short-term improvement of stroke (Kasner et al., 2002). As seen from the above-mentioned studies, there is currently no concrete evidence from randomized trials to support the routine use of chemical or physical cooling therapy in acute stroke. Nevertheless, since animal studies have shown a neuroprotective effect of hypothermia in cerebral ischemia, further trials with cooling therapy in acute stroke are warranted.

However, keeping all this in perspective, thrombolytic therapy with tPA is the only scientifically proven effective therapy for acute ischemic stroke (Albers 1999; Traynelis and Lipton, 2001) and is the front-line treatment for acute stroke in the clinical setting barring any contraindications, such as evidence of hemorrhage.

Thrombolytic therapy

Thrombolytic therapies aim to disintegrate the emboli responsible for cerebral artery occlusion, thus restoring blood flow to ischemic brain regions. As mentioned above, presently tissue plasminogen activator (tPA) is the only approved thrombolytic therapy for acute stroke (Albers 1999; Traynelis and Lipton, 2001). This protein activates the

enzyme plasminogen, converting it to plasmin. Plasmin disintegrates fibrin, the principal component of an embolus. Administration of tPA therefore results in destruction of emboli and resumption of blood flow through occluded arteries. Although other promising treatments exist, clinical trials have yet to show any significant improvement to warrant their use by the medical community.

The effectiveness of tPA treatment has been demonstrated by the well known clinical trials held by The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (NINDS, 1995). The major drawback to tPA treatment is the risk of cerebral hemorrhage associated with its use. This risk eliminates any benefit if tPA is administered more than three hours after stroke onset (Hacke et al., 1995). For a patient to be eligible for tPA treatment, stroke onset must be less than three hours prior to tPA administration and intracranial bleeding has to be ruled out. There is no information on the effectiveness of using tPA in acute stroke in the presence of hyperthermia.

When an artery is occluded, not only is neural tissue affected, the vasculature (including the BBB) also undergoes degenerative changes (Hamann et al., 1995; Fujimura et al., 1999). These changes explain the observed increase in vascular permeability as measured by a protein tracer (Dietrich et al., 1990b) and Evan's blue dye (Belayev et al., 1998). The increased vascular permeability of the BBB could be one of the reasons why thrombolytic treatment is associated with a risk for hemorrhagic transformation (del Zoppo et al., 1998). As mentioned, tPA converts plasminogen to plasmin. It has been shown that plasmin cleaves laminin (Liotta et al., 1981), an integral component of the

basal lamina (a constituent of vasculature). Thus, tPA degrades the BBB that has already become vulnerable due to ischemia. Since hyperthermia during stroke may also influence dysfunction of the BBB, tPA treatment of acute stroke superimposed with hyperthermia could result in severe consequences, which remain to be examined.

The need and justification for tPA use arises from the fact that ischemic strokes are the most common type of acute strokes in the clinical setting. Approximately 50% of strokes are caused by cerebral thrombosis and another 30% result from cerebral embolism. Thromboembolism is involved in 80 – 90% of stroke cases (Mohr et al., 1978; Sloan 1987) and the majority of ischemic episodes occur as a result of occlusion of the middle cerebral artery (MCA) or its branches (Wang et al., 2001a). After an ischemic event, neuronal death evolves in a time-dependent fashion determined by both the duration and severity of blood flow (Overgaard et al., 1992; Siesjo 1992). The duration of blood flow interruption is the most important determinant of infarct size (Wang et al., 2001b). As the majority of ischemic strokes are caused by thrombotic or thromboembolic arterial occlusions, therapeutic strategies designed to restore cerebral perfusion hold great promise for these patients.

Delayed reperfusion, on the other hand, can lead to negative sequelae such as the breakdown of the BBB and the development of hemorrhagic transformation and edema (Aoki et al., 2002), all of which may be further aggravated by the presence of hyperthermia.

Following a stroke, an infarcted brain appears pale initially; within hours to days, dilated blood vessels and minute petechial hemorrhages are apparent in the gray matter. Reestablishment of circulation in the infracted area by embolus migration, lysis, or dispersion can cause further injury due to the possibility of hemorrhagic transformation. Therefore, the most effective therapy for stroke is prevention as the brain is unable to repair itself, forming only a fibrogliotic scar tissue at the site of infarction. After a stroke, therapeutic drive is towards minimizing subsequent worsening of the infarction and preventing a second stroke.

Secondary stroke prevention

Stroke prevention, particularly in patients who have already experienced stroke or TIA, is critical for reducing the burden of disease on patients, their families, and society. The notion of secondary stroke prevention is based on the premise that once stroke has occurred and stabilized, not much else can be done in terms of treatment and the focus of stroke management shifts from treatment to secondary stroke prevention which includes combating risk factors associated with stroke and maintenance of healthy vasculature. Therapies designed to improve vascular health can, therefore, greatly diminish the risk for developing future ischemic events.

Endothelial dysfunction and cerebrovascular disease

Traditionally, the endothelium has been considered an inert component of the vessel wall but over the last two decades it has become evident that it plays a crucial role in regulating vascular tone and structure (Landmesser et al., 2004a). Vascular endothelial

cells form a structurally simple but functionally sophisticated organ that is involved in regulating biological processes as diverse as fibrinolysis, inflammation, hemastasis, lipoprotein metabolism, blood pressure, and angiogenesis (O'Connell and Genest 2001). Given its unique anatomical position, the endothelium is a primary target for mechanical and biochemical injuries caused by traditional risk factors such as hypertension, hyperlipidemia, diabetes mellitus, and smoking. Alterations in one or more of the physiological roles of the endothelium constitute endothelial dysfunction. Injury to the endothelium results in deleterious alterations of endothelial physiology, which represent a key early step in the development of an atherosclerotic lesion and are implicated in plaque progression and destabilization, thrombus generation, and cardiac and cerebral ischemic injury (Calabresi et al. 2003).

The healthy endothelium acts to maintain vascular homeostasis through multiple complex interactions with cells in the vessel wall and lumen. Table 1.1 lists some of the major factors regulated by the vascular endothelium (Gokce and Vita 2002). Specifically, the endothelium regulates vascular tone by balancing production of vasodilators, including NO, and vasoconstrictors. Endothelium also controls blood fluidity and coagulation by producing factors that regulate platelet activity, the clotting cascade, and the fibrinolytic system. Endothelium can also produce cytokines and adhesion molecules that are involved in regulation of the inflammatory process (Libby et al., 2002).

Table 1.1: Factors regulated by the endothelium to maintain vascular homeostasis

Maintenance of vascular tone
Nitric Oxide
Prostaglandins
Endothelin – 1
Angiotensin II
Regulation of vascular inflammatory process
Monocyte chemotactic factor – 1
Adhesion molecules
Interleukins 1, 6, and 18
Tumor necrosis factor
Regulation of hemostasis
Nitric Oxide
Tissue plasminogen activator
Heparins
Prostaglandins
Thrombomodulin
Plasminogen activator inhibitor – 1
Tissue factor
Von Willibrand's factor

The integrity and functional activity of the endothelial monolayer is required for the maintenance of normal vessel wall properties. Under homeostatic conditions, the endothelium maintains normal vascular tone and blood fluidity, and there is little or no expression of pro-inflammatory factors. However, common conditions predisposing to atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking can initiate a chronic proinflammatory and prothrombotic phenotype of the endothelium. These conditions are associated with the loss of endothelium-dependent vasodilation in both adults and children (Celermajer et al., 1992; Sorensen et al., 1994; Gokce and Vita 2002). More recently, risk factors such as obesity (Steinberg et al., 1996), elevated C-reactive protein (Fichtlscherer et al., 2000), and chronic systemic infection (Prasad et al., 2002) have also been associated with endothelial dysfunction.

Endothelial dysfunction is usually one of the earliest markers of atherosclerosis, and predisposes to vasoconstriction and thrombosis (Verma and Anderson 2002). Injury to the endothelium by either mechanical disruption or inflammatory activation of the endothelial cells induces a cascade of proinflammatory events resulting in infiltration of monocytic cells and smooth muscle proliferation (Libby et al., 2002). These processes can lead to development and clinical expression of atherosclerosis (Levine et al., 1995). The maintenance of endothelial integrity is therefore of crucial importance in preventing the triggering of these processes. Atherosclerotic plaques generally begin to form between the ages of 20 and 30 years, when a person is asymptomatic. As mentioned above, inflammation at the endothelial level is usually the main trigger and as leukocytes localize in early atherosclerotic lesions, they attach to the vascular endothelium and travel

to the intima, initiating a perpetual inflammatory response. Monocytes evolve into macrophages, which scavenge for lipids and eventually mature into foam cells. T lymphocyte expression of inflammatory cytokines is also increased, which activates macrophages and endothelial cells, propagating smooth muscle cells and narrowing the vasculature. Maturing macrophages produce enzymes that degrade the fibrous cap, making it vulnerable to rupture, and also produce procoagulant tissue factor, which contributes to the development of thrombosis (Viles-Gonzalez et al., 2004). As inflammation continues, elevated levels of C-reactive protein inhibit NO release, promoting monocyte adhesion to the endothelium and increasing low-density lipoprotein uptake into the plaques (Rost et al., 2001).

Given the possible casual pathway from endothelial dysfunction to atherosclerosis (Figure 1.1), numerous methods have been employed to measure endothelial dysfunction in humans. Endothelial function has largely been assessed as the ability of an artery to vasodilate in response to a stimulus that causes NO release in healthy blood vessel (O'Connell and Genest 2001). Such stimuli include shear stress from increased blood flow and receptor-dependent agonists, such as acetylcholine, bradykinin, or substance P. NO, synthesized by the endothelial NO synthase (eNOS) from the precursor L-arginine, is not only a major mediator of endothelium-dependent vasodilation but is also involved in regulation of other protective properties of the healthy endothelium (Landmesser et al., 2004b).

Figure 1.1: Role of endothelial dysfunction in development of vascular disease

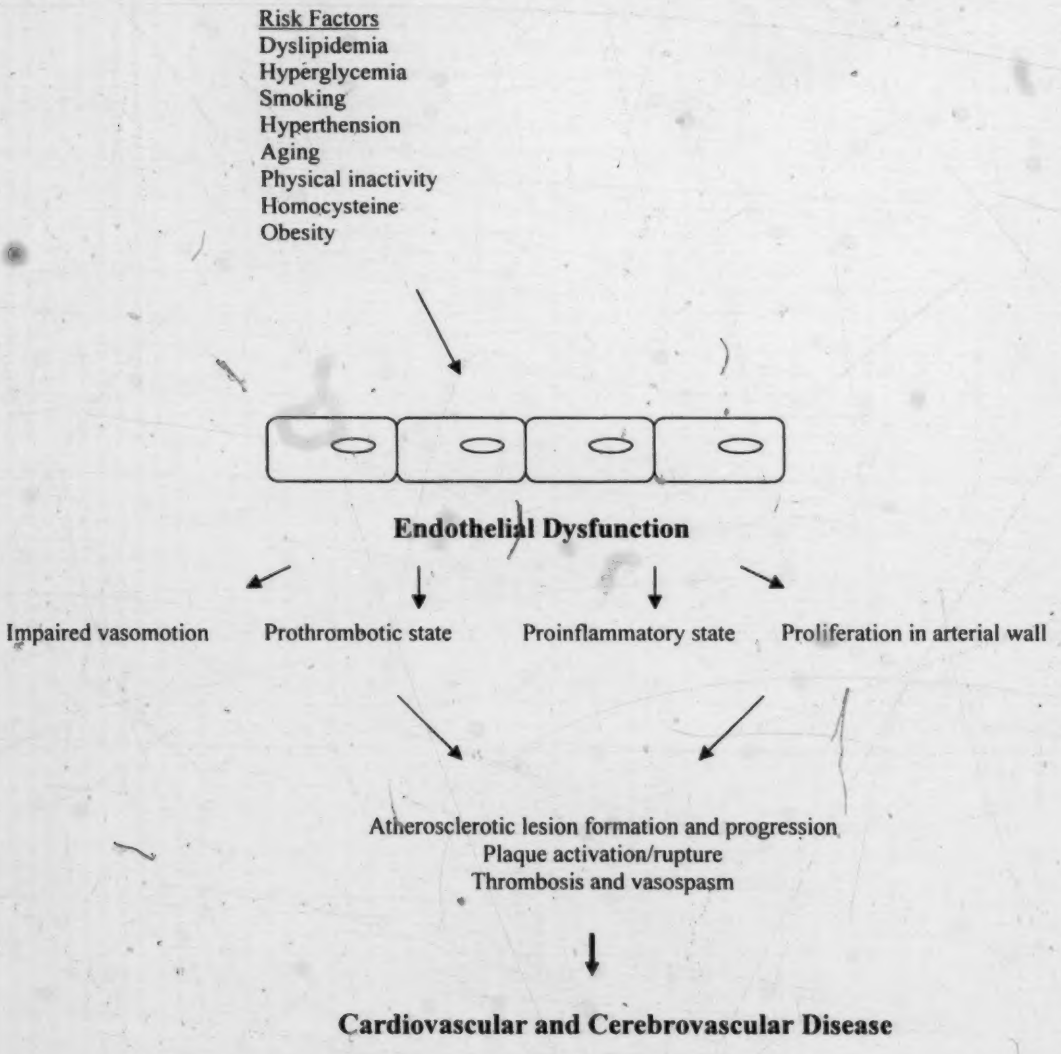

Figure 1.1: Vascular disease risk factors adversely affect a number of endothelial homeostatic functions and contribute to the development, progression, and clinical expression of atherosclerosis (Adapted from Widlansky et al., 2003).

As mentioned above, inflammation plays a pivotal role in all stages of atherosclerosis, including initiation, progression and clinical expression. There is increasing evidence for the anti-inflammatory role of endothelium-derived NO. NO prevents endothelial adhesion of monocytes and chemokine expression and inhibits thrombocyte activation and aggregation, thereby exerting important anti-inflammatory and antithrombotic effects. Pharmacologic inhibition of endothelium-derived NO production leads to a marked increase in endothelial adhesiveness for monocytes (Tsao et al., 1994; De Caterina et al., 1995). This concept has been supported by observations in eNOS-deficient mice that have a marked increase in leukocyte-endothelial cell interactions and acceleration of atherosclerotic lesion development (Lefer et al., 1999; Kuhlencordt et al., 2001). In mice lacking the low-density lipoprotein (LDL) receptor, an animal model of hypercholesterolemia, inhibition of endothelial NO production accelerates atherosclerotic lesion formation, whereas treatment with L-arginine, precursor for NO, decreases lesion development (Aji et al., 1997). In apolipoprotein E (apoE)-knockout mice that are prone to developing atherosclerosis, specific removal of eNOS results in a marked acceleration of coronary atherosclerosis (Kuhlencordt et al., 2001).

Apart from its effects on leukocyte adhesion, endothelium-derived eNOS also limits platelet activation, adhesion, and aggregation (Loscalzo 2001). It also produces antithrombotic effects through inhibition of expression of plasminogen activator inhibitor-1, a prothrombotic protein (Bouchie et al., 1998). Since NO is a particularly important mediator of endothelium-dependent vasodilation and anti-inflammatory and

antithrombotic effects of the endothelium, endothelium-dependent vasomotion is thought to represent an accessible indicator of endothelial health.

Endothelial function in patients

In clinical practice, endothelial function is measured indirectly by measuring flow-dependent dilation of the brachial artery in response to a stimulus that causes NO release in a healthy blood vessel. The measurement of flow-dependent dilation of the brachial artery as a noninvasive endothelial function test was introduced by Celermajer et al. in 1992. This approach has now been used by numerous groups throughout the world to monitor endothelial function. The method involves increases in laminar shear stress, the tangential force exerted by blood flow over the surface of the endothelium. Increases in shear stress lead to a rapid activation of eNOS which, over a longer period, leads to increased expression of eNOS (Traub and Berk 1998). Flow-dependent dilation of the radial and brachial arteries is largely inhibited after NO synthase inhibition in humans (Hornig et al., 1996). Abnormalities in flow-mediated brachial reactivity are associated with an increased risk of cardiovascular events, while coronary endothelial dysfunction predicts not only future cardiovascular, but also cerebrovascular events, demonstrating the systemic nature of the atherosclerotic process (Perticone et al., 2001; Halcox et al., 2002; Targonski et al., 2003)

Endothelial dysfunction and endothelial progenitor cells

More recently, it has been suggested that circulating endothelial progenitor cells (EPCs) may also be a marker of endothelial function and cardiovascular risk. In a recent study,

the numbers of circulating endothelial progenitor cells was related to flow-dependent dilation of the brachial artery and to the cardiovascular risk factor score. In healthy men, decreasing numbers of EPCs were strongly correlated with increased Framingham risk score, while a higher number of EPCs were correlated with improved endothelial function, as measured by flow-mediated brachial reactivity (Hill et al., 2003). Evidence continues to accumulate on the importance of EPCs in neovascularization of ischemic tissues and inhibition of neointimal proliferation (Asahara et al., 1999; Iba et al., 2002). EPCs isolated from circulating blood may originate from bone marrow (Asahara et al., 1997; Lin et al., 2000) or from resident EPCs embedded within organs (Majka et al., 2003). In contrast to differentiated mature endothelial cells, EPCs have a high proliferation potential and can be expanded extensively *in vitro* (Asahara et al., 1997; Simper et al., 2002). According to the initial discovery, EPCs were defined as cells positive for both hematopoietic stem cell markers such as CD34 and vascular endothelial growth factor receptor 2 (VEGFR2), an endothelial marker protein. However, more recent studies have also used CD133, a more immature hematopoietic cell marker (Peichev et al., 2000). Transplantation of EPCs enhances vascular development by in situ differentiation and proliferation within ischemic organs (Kalka et al., 2000a). The number of circulating EPCs is significantly downregulated in patients with endothelial dysfunction and coronary artery disease (Vasa et al., 2001a). EPC numbers are significantly decreased in subjects with elevated serum cholesterol, hypertension and diabetes (Hill et al., 2003) and in smokers (Perticone et al., 2001), while EPC migratory ability is also impaired by hypertension (Vasa et al., 2001a).

EPCs and endothelial repair

After the initial report (Asahara et al., 1997), there has been substantial interest in understanding the mobilization, homing, and function of EPCs in various conditions. Circulating EPCs may represent an important endogenous repair mechanism to maintain the integrity of the endothelial monolayer and promote ischemia-induced neovascularization. Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia (Isner and Asahara 1999). In animal models of myocardial infarction, the injection of ex vivo expanded EPCs significantly improved blood flow and cardiac function and reduced left ventricular scarring (Kawamoto et al., 2001; Kocher et al., 2001). Similarly, infusion of ex vivo expanded EPCs in nude mice or rats improved neovascularization in hind limb ischemia models (Kalka et al., 2000a; Urbich et al., 2003). Remarkably, terminally differentiated mature endothelial cells did not improve neovascularization (Kalka et al., 2000a). Clinical pilot trials also indicate that bone marrow-derived or circulating blood-derived EPCs are useful for therapeutically improving blood supply of ischemic tissue (Assmus et al., 2002; Tateishi-Yuyama et al., 2002). Transplantation of ex vivo expanded EPCs significantly improved coronary flow reserve and left ventricular function in patients with acute myocardial infarction (Assmus et al., 2002).

Although the role of EPCs in neovascularization has been shown by several groups now, it is still not clear as to how they improve neovascularization. The extent of incorporation of bone marrow-derived cells in cerebral vessels after stroke varies in literature. Two studies reported positive vessels with an average of 34% endothelial

marker expressing bone marrow cells (Hess et al., 2002; Zhang et al., 2002), whereas another group only reported a minor contribution by bone marrow-derived EPCs to vascularization in murine gliomas (Machein et al., 2003). The number of incorporated cells with an endothelial phenotype into ischemic tissues is generally quite low. Therefore, the efficiency of neovascularization may not only be due to the incorporation of EPCs in newly formed vessels but may also be influenced by release of proangiogenic factors in a paracrine manner (Urbich and Dimmeler 2004).

In the past, the turnover of endothelial cells was believe to be very low (Caplan 1973); however, it is now understood that cardiovascular risk factors increase endothelial cell apoptosis, resulting in a disturbance of the endothelial monolayer (Dimmeler et al., 2002). Risk factors for atherosclerosis can induce a proinflammatory response in endothelial cells and directly induce endothelial injury by promoting cellular apoptosis. Risk factors such as proinflammatory cytokines, reactive oxygen species, oxidized LDL, and angiotensin II have all been shown to promote endothelial apoptosis (Dimmeler et al., 2002). In the past, the regeneration of injured endothelium was attributed to the migration and proliferation of neighboring endothelial cells. Recently, it has been suggested that the injured endothelial monolayer may also be regenerated by circulating endothelial cells (Figure 1.2).

Figure 1.2: Regeneration of endothelial monolayer by EPCs

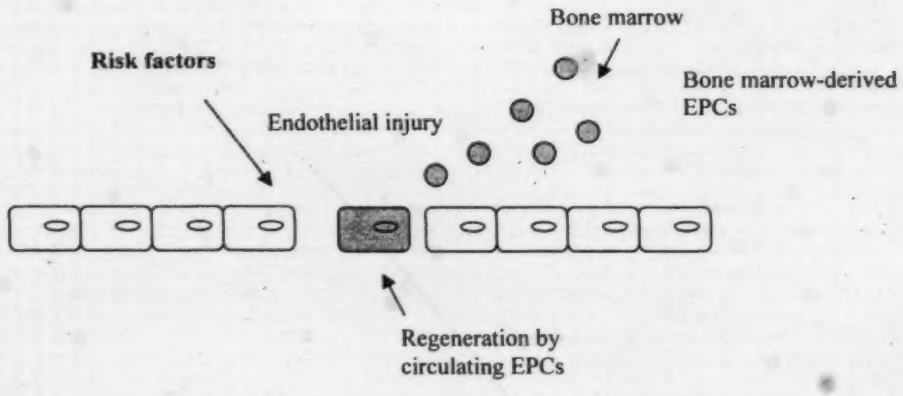

Figure 1.2: In the past endothelial regeneration after injury was attributed mainly to the migration and proliferation of neighboring mature endothelial cells. However, recently many studies have reported that the injured endothelial monolayer may also be repaired by bone-marrow derived EPCs.

In a dog model, implanted Dacron grafts were shown to be rapidly covered by bone marrow-derived $CD34^+$ hematopoietic stem cells (Shi et al., 1998). A similar effect was also seen in humans, where surfaces of ventricular assist devices were found to be covered with $CD133^+$ and VEGFR2 hematopoietic stem cells (Peichev et al., 2000). Circulating EPCs were also reported to home to denuded parts of an artery after balloon injury (Walter et al., 2002). Enhanced incorporation of bone marrow-derived cells was associated with accelerated reendothelialization and reduced restenosis. The incorporated cells were shown, using mice that had bone marrow transplantation with β-galactosidase-expressing cells, to derive from the bone marrow (Walter et al., 2002; Werner et al., 2002). The regenerated endothelium was functionally active, as shown by the release of NO (Fujiyama et al., 2003). These studies imply that endothelial cells may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery. They may also regenerate the continual low grade endothelial damage caused by ongoing induction of endothelial apoptosis induced by cardiovascular risk factors (Rossig et al., 2001). The maintenance of the endothelial monolayer may prevent thrombotic complications and atherosclerotic lesion development since transplantation of $ApoE^{-/-}$ mice with wild-type bone marrow was reported to reduce atherosclerotic lesion formation (Rauscher et al., 2003). Classical risk factors for coronary artery disease, such as diabetes, hypercholesterolemia, hypertension, age, and smoking, affect the number and functional activity of EPCs in healthy volunteers (Hill et al., 2003) and in patients with coronary artery disease (Vasa et al., 2001a). The reduction in EPC numbers by risk factors may contribute to a vicious cycle, resulting in aggravation of endothelial dysfunction

(Dimmeler and Zeiher 2004). Because EPCs contribute to reendothelialization and neovascularization, increasing their numbers may be an attractive therapeutic tool. Factors that reduce cardiovascular risk such as statins (Llevadot et al., 2001; Walter et al., 2002) or exercise (Laufs et al., 2004) elevate EPC levels, which contributes to enhanced endothelial repair. EPC levels and their capacity for endothelial repair may therefore be influenced by the balance between atheroprotective and proatherosclerotic factors.

Regulation of EPC levels

Since impaired circulating EPC numbers and/or function appear to contribute to atherosclerotic disease progression in patients with risk factors for coronary artery disease, their augmentation may be an important therapeutic approach to improving patient health. The mobilization of hematopoietic stem cells in the bone marrow is determined by the local microenvironment, which consists of endothelial cells, osteoblasts, and fibroblasts (Papayannopoulou 2004). Mobilizing cytokines weaken the interactions between stem cells and stromal cells, which finally allow the stem cells to leave the bone marrow via transendothelial migration. This requires activation of proteinases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs) which cleave adhesive bonds on stromal cells that interact with integrins on hematopoietic cells (Heissig et al., 2002). Physiologically, ischemia is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow by upregulating vascular endothelial growth factor (VEGF) or stromal cell derived factor-1 (SDF-1), which in turn are released to the circulation and induce mobilization of progenitor cells from the bone marrow (Pillarisetti and Gupta 2001; Heissig et al., 2002). VEGF gene transfer in a

human clinical study has also been reported to augment circulating EPC levels, providing support for VEGF-induced mobilization of progenitor cells from the bone marrow (Kalka et al., 2000b). Additional factors for mobilization of progenitor cells from the bone marrow include granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte-colony stimulating factor (GM-CSF), and erythropoietin (Takahashi et al., 1999; Heeschen et al., 2003).

Initial evidence for potential pharmacological modulation of systemic EPC levels by atheroprotective drugs came from studies using 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). Statins were shown to increase the number and functional activity of EPCs *in vitro*, in mice, and in patients with coronary artery disease (Dimmeler et al., 2001; Llevadot et al., 2001; Vasa et al., 2001b). The mechanisms for statin-induced increase in EPC numbers are not clear; however, they may include an increase in proliferation, mobilization, and prevention of EPC senescence and apoptosis (Dimmeler et al., 2001; Llevadot et al., 2001; Assmus et al., 2003). Recently, estrogen was shown to increase the levels of circulating EPCs (Strehlow et al., 2003). Similarly, exercise is also reported to augment EPC levels in mice and humans (Laufs et al., 2004). The molecular signaling pathways have not been identified thus far. However, studies indicate that the activation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway, which has been shown to be activated in mature endothelial cells by statins (Kureishi et al., 2000) may also be involved in statin-induced increase in EPC levels (Assmus et al., 2003). Physical exercise and other factors which increase EPC levels, such as estrogen and erythropoietin are well established activators of the PI3K/Akt

pathway, suggesting that this may be a common pathway to increase EPC levels (Dimmeler and Zeiher 2004). Interestingly, the mobilization of EPCs by VEGF, statins, estrogen, or physical exercise has been shown to be dependent on eNOS (Aicher et al., 2003; Iwakura et al., 2003; Landmesser et al., 2004b; Laufs et al., 2004). These stimuli may increase EPC mobilization by PI3K/Akt-dependent activation of the NO-synthase in the bone marrow stromal cells.

Reduction in EPC numbers by cardiovascular risk factors

The reduction in circulating EPC numbers by cardiovascular risk factors may be as a result of exhaustion of the pool of stem/progenitor cells in the bone marrow, reduced mobilization of progenitor cells, or increase in apoptosis of EPCs. The direct influence of cardiovascular risk factors on the bone marrow microenvironment is not clear. However, aging has been shown to decrease stress-induced mobilization of stem/progenitor cells from the bone marrow (Geiger and Van Zant 2002). Elderly patients are reported to show a decrease in circulating EPCs following coronary artery surgery (Scheubel et al., 2003). Age may also interfere with the functional activity of stem/progenitor cells since transplantation of young bone marrow-derived cells restored age-associated impaired neovascularization, while bone marrow of aged mice was not effective (Edelberg et al., 2002). Young bone marrow-derived cells also provide protection against atherosclerotic lesion formation (Rauscher et al., 2003).

Since cardiovascular risk factors systemically impair endothelial cell functions, they may also influence functional activity of the bone marrow resident stromal cells which also

partially consist of endothelial cells. Indeed, exercise and VEGF-stimulated EPC mobilization were reported to be blunted in eNOS-deficient mice (Aicher et al., 2003), and these researchers showed a reduced neovascularization capacity in the hind limb ischemia or retina ischemia model, suggesting that eNOS-dependent signaling is important for EPC function (Aicher et al., 2003; Guthrie et al., 2005). Bone marrow-derived cells from patients with chronic heart failure showed a reduced migratory response ex vivo and significant impairment to home to sites of ischemia and to improve neovascularization after hind limb ischemia (Heeschen et al., 2004). Since NO bioavailability is reduced in patients with coronary artery disease and heart failure, it is possible that this reduction may lead to reduced mobilization of EPCs.

Enhanced EPC apoptosis may also underlie the reduction in EPC levels by cardiovascular risk factors. Coronary artery disease is associated with an imbalance in pro- and anti-apoptotic factors and a decline in antioxidant defense which may increase EPC susceptibility to apoptosis and increase their turnover rate (Dimmeler and Zeiher 2004). Statins and estrogen-induced increases in EPC levels are associated with inhibition of EPC apoptosis (Llevadot et al., 2001, Strehlow et al., 2003) whereas angiostatin, an antiangiogenic molecule, was shown to block proliferation of EPCs (Ito et al., 1999). Such findings show that cardiovascular risk factors might interfere with EPC-mediated vascular protection, thereby modulating endothelial repair process. Improvement of EPC levels or function by pharmacological interventions could therefore be an attractive novel therapeutic option for antiatherosclerotic therapy.

High-density lipoprotein cholesterol and vascular function

Cholesterol is a key molecule in the atherogenic process. A high level of LDL cholesterol is one of the most significant causes of cardiovascular disease. Large clinical trials using HMG-CoA reductase inhibitors in subjects with elevated LDL levels have demonstrated marked improvement in clinical outcomes (Shepherd et al., 1995; Sacks et al., 1996). However, despite the important beneficial effects of these medications, a large number of cardiovascular events still occur in those patients who are effectively treated for high LDL (Young et al., 2004). Several epidemiological studies have provided evidence that a low level of high-density lipoprotein (HDL) cholesterol is a major, independent risk factor for the development of coronary heart disease (Corti et al., 1995; Goldbourt et al., 1997). Therefore, there is in increasing interest in understanding the effects of HDL cholesterol on vascular function.

HDLs are a heterogeneous group of small, dense lipoproteins that are isolated from human plasma at a density of 1.063 to 1.125 g/mL. Approximately 50% of HDL particle mass is composed of proteins while the remainder is represented by lipids. Most HDL particles contain apolipoprotein A-I (apoA-I) as the major protein component. Several other proteins, including apoA-II, apoA-IV, apoE, and lecithin cholesterol acyltransferase are also associated with HDL and impart significant physiological roles (Silverman et al., 1993; Calabresi et al., 2003).

Endothelial cells are continuously exposed to substances in the blood that exert a number of regulatory functions on endothelial cells in an endocrine, paracrine, and autocrine

fashion. Plasma lipoproteins such as LDL and HDL have been shown to alter the endothelium *in vivo* in both the long and short term. Patients with elevated LDL cholesterol have abnormal vasodilatory function in response to flow-mediated dilation and exhibit a higher risk of development of atherosclerosis. HDL, on the other hand, is atheroprotective and appears to modulate endothelial function in a beneficial fashion (Gotto 2001). Studies in patients with rare disorders of HDL metabolism and in knock-out animal models support a casual relationship between low HDL and development of atherosclerotic vascular disease. The atheroprotective activity of HDL is often explained by the ability of these lipoproteins to remove cholesterol from peripheral tissues, including the arterial wall, and transport it to the liver for excretion in the bile. This important process, termed "reverse cholesterol transport" was initially described by Glomset et al. in 1966.

Endothelial dysfunction has been reported in patients with primary hypoalphalipoproteinemia, a genetic disorder characterized by low plasma HDL cholesterol and apoA-I levels and high coronary heart disease risk (Calabresi et al., 2002; Vaudo et al., 2003). Low plasma HDL concentration is an independent risk factor of endothelial dysfunction in healthy individuals and hyperlipidemic and diabetic patients (Zhang et al., 2000; Chan et al., 2001; Lupattelli et al., 2002). Elevation of plasma HDL levels by drug treatment or infusion of synthetic HDL leads to a significant improvement in impaired endothelial function (Kuvin et al., 2002; Spieker et al., 2002). In addition to the reverse cholesterol pathway, HDL has additional roles in the prevention of vascular diseases (Figure 1.3).

Figure 1.3: Effects of HDL on endothelial cells

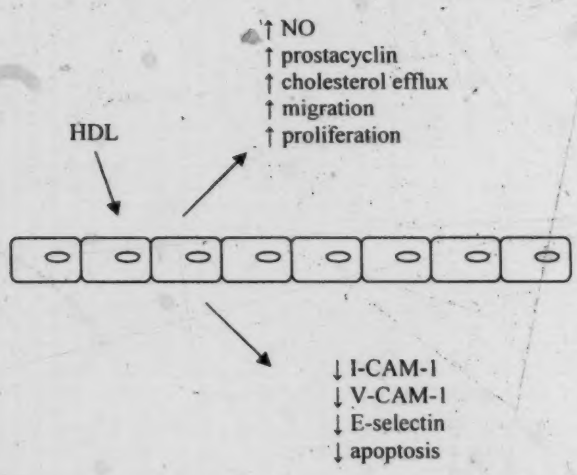

Figure 1.3: HDL has various effects on the vascular endothelial cells, including increased release of NO and prostacyclin, which lead to increased smooth muscle cell relaxation and vasodilation. HDL also inhibits expression of endothelial adhesion molecules, thereby inhibiting initiation of inflammatory processes.

Oxidized LDL particles are potent inducers of endothelial dysfunction through involvement in the inflammatory process that occurs in the vessel wall during atherogenesis. Protective effects of HDL on endothelial function may be attributable in part to their capacity to counteract the deleterious effects of oxidized LDL as HDL has been shown to have an inhibitory effect on the rate of macrophage-induced oxidation of LDL (Parthasarathy et al., 1990). HDL also exerts an anti-inflammatory effect in the vasculature by inhibiting LDL-induced upregulation of the cell adhesion molecules such as, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (Cockerill et al., 1995). Early in the pathogenesis of atherosclerosis, endothelial cells display an increased affinity for leukocytes, which adhere to and migrate through the endothelium, starting the process of atherogenesis. Inflammatory cytokines, such as TNF-α and IL-1β also induce endothelial expression of leukocyte adhesion molecules (Dart and Chin-Dusting 1999). A number of studies have shown that HDL attenuates the cytokine-induced expression of the adhesion molecules VCAM-1 and ICAM-1, and of E-selectin in cultured endothelial cells (Cockerill et al., 1995). Animal experiments show that reconstituted HDL inhibits endothelial VCAM-1 expression and neointima formation in a mouse model of carotid artery injury (Dimayuga et al., 1999).

Additionally, HDL upregulates prostacyclin (prostaglandin I_2), which inhibits platelet activation and vascular smooth muscle cell growth and migration (Cockerill et al., 1999). Prostacyclin increases synthesis of NO by endothelial cells, and in turn, NO increases the activity of prostacyclin on smooth muscle relaxation. Incubating endothelial cells with

HDL promotes prostacyclin synthesis (Fleisher et al., 1982) and HDL also prolongs the half-life of prostacyclin in the blood (Yui et al., 1988). HDL also decreases complement activation and increases intracellular ceramide by inhibiting sphingosine kinase, thereby reducing inflammation (Xia et al., 1999). These antioxidant and anti-inflammatory properties of HDL could help to ameliorate the initial steps of plaque formation and also limit thrombotic potential (Young et al., 2004).

Recent data also indicate that HDL has direct beneficial effects on the vascular endothelium. Inhibition of endothelium-dependent relaxation due to a decrease in NO bioavailability is the most prominent feature of endothelial dysfunction. In the endothelial cell, NO is generated by eNOS, which is primarily localized to caveolae, cholesterol-rich microdomains of the plasma membrane containing a variety of signal transduction molecules. eNOS becomes activated in response to a number of stimuli, including fluid shear stress and exposure to diverse agonists of G-protein-coupled receptors (O'Connell and Genest 2001). High HDL in humans is associated with normal endothelium-dependent vasorelaxation in response to acetylcholine (Watts et al., 1996), whereas LDL is associated with a decrease in acetylcholine-induced endothelium-dependent relaxation or even a paradoxical vasoconstrictive response (Vita et al., 1990). Incubation of endothelial cells with oxidized LDL depletes plasma membrane caveolae of cholesterol and translocates eNOS from caveolae to an internal membrane compartment, thus making eNOS insensitive to stimulation by acetylcholine (Blair et al., 1999). HDL prevents this defective localization of eNOS by donating cholesterol esters to endothelial cell caveolae (Uittenbogaard et al., 2000). Incubation of cultured mature endothelial cells

with HDL activates eNOS by the binding of apoA-I to the scavenger receptor-BI (Yuhanna et al., 2001). Prolonged exposure to HDL also enhances eNOS expression in cultured human endothelial cells (Kuvin et al., 2002). Clinical studies show that HDL prevents endothelial dysfunction by promoting endothelial NO production. Plasma concentration of HDL is reported to be an independent predictor of NO-dependent peripheral vasodilation in healthy individuals (Chan et al., 2001), hyperlipidemic and diabetic patients (Lupattelli et al., 2002), and patients with coronary artery disease (Zhang et al., 2000). Peripheral endothelial function improves in patients with established cardiovascular disease when low HDL levels are increased by niacin (Kuvin et al., 2002).

Endothelium integrity as a continuous and intact monolayer of endothelial cells is essential for the maintenance of normal vessel wall properties. This monolayer may be transiently or chronically disrupted by endothelial cell turnover, traumatic injury, or pathological damage (Calabresi et al., 2003). Apoptosis of endothelial cells has been demonstrated in many cardiovascular diseases, including atherosclerosis. Many atherogenic lipids and inflammatory cytokines associated with endothelial dysfunction are potent apoptotic stimuli for endothelial cells (O'Connell and Genest 2001). Endothelial cell apoptosis can lead to increased permeability of vascular endothelium, leukocyte adhesion to the vessel wall, smooth muscle cell proliferation, and enhanced blood coagulation. An intact endothelium is essential to mask the thrombogenic molecules that lie underneath the vessel wall (Calabresi et al., 2003). HDL, at physiological concentrations, protects cultured human endothelial cells from TNF-α-

induced apoptosis in a dose-dependent manner, and this effect is mediated through inhibition of caspase-3 activity (Sugano et al., 2000). HDL also suppresses the mitochondrial pathway of apoptosis, which is induced by growth factor deprivation. This requires activation of Akt, an antiapoptotic protein kinase that maintains mitochondrial integrity, thus inhibiting cytochrome c release, and activation of caspase 3 and 9 (Nofer et al., 2001).

There is accumulating evidence that a close association exists between HDL, endothelial dysfunction, and possibly EPC levels in relation to cardiovascular events. Alterations in endothelial and EPC function may play an important pathophysiological role and promote development and complications of vascular disease. The capacity of HDL to prevent and correct endothelial dysfunction through various mechanisms in mature endothelial cells warrants an examination of its effects on EPCs which are also suggested to be a marker of endothelial function and cardiovascular risk.

Chapter 2: Animal Models of Cerebral Ischemia

To test the potential efficacy of any drug on stroke patients, an animal model of stroke must be initially used to gather enough experimental data on the drug's efficacy for it to be able to be approved for clinical trials. Ideal models of cerebral ischemia in animals must closely resemble the clinical situations. When compared to gerbils, cats, and dogs, the rat cranial circulation is most similar to that of the human (Yamori et al., 1976). A rat model of stroke has been used extensively in our lab to investigate various mechanisms of cerebral ischemia and the efficacy of a variety of therapeutic treatments (Wang et al., 2001c).

The MCA is believed to be the most commonly blocked artery in embolic stroke (Brust 1991). Thus the ideal model for these experiments was a rat model of MCA occlusion. However, as will be shown below, there are many models of MCA occlusion in the rat, and selection of an appropriate model greatly influences the kinds of studies one can conduct and also reliability and accuracy of data one can gather.

Some models of stroke in rats involve permanent occlusion of the MCA. One model of permanent MCA occlusion in rats was established by Tamura et al. in 1981. This model required the MCA to be exposed prior to being occluded; however, the authors did not specify exactly how the MCA was occluded (Tamura et al., 1981). Unfortunately, permanent MCA occlusion (MCAo) does not accurately mimic the typical clinical situation. Spontaneous recanalization occurs in up to 50% of cases involving stroke patients suffering from MCA occlusion (Saito et al., 1987). Since the present study was

designed to evaluate the efficacy of thrombolytic treatment in stroke during hyperthermia, a model that allowed reperfusion to take place would thus be more useful.

The most commonly used models of global and focal cerebral ischemia are produced by surgical occlusion or insertion of a filament into the common carotid or the internal carotid arteries. The most popular model in use today is the intraluminal filament model, first described by Koizumi and colleagues (Koizumi et al., 1986). In this model, a nylon filament (coated with poly-L-lysine) is advanced up the internal carotid artery all the way up to a few millimeters beyond the MCA (Figure 2.1). Infarction size can be controlled by the length of time the nylon thread is left inside. However, although reperfusion can be established following removal of the thread, this model, does not recapitulate the events that occur in human stroke and cannot be used for evaluation of thrombolytic therapy. Another rat model of reversible MCAO is prepared by application of endothelin-1 (a very potent vasoconstrictor) to the exposed MCA (Robinson et al., 1990). Endothelin causes vasoconstriction of the MCA for a period of time, followed by relaxation allowing for reperfusion. The dose of endothelin applied affects the strength and duration of MCA vasoconstriction, and thereby affects the severity of infarction. Other methods of inducing ischemia followed by reperfusion involve the temporary occlusion of arteries using surgical clips (Dietrich et al., 1989), hooks (Kaplan et al., 1991) or ligature snares (Shigeno et al., 1985).

Figure 2.1: Intraluminal filament model of MCA occlusion

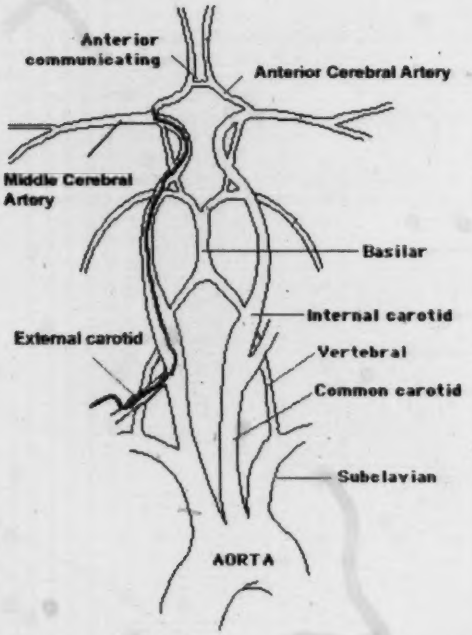

Figure 2.1: In the intraluminal filament model of MCA occlusion, a nylon filament is introduced into the internal carotid artery via the external carotid artery and extends past the middle cerebral artery, compromising blood flow to the brain areas irrigated by the middle cerebral artery.

While it is useful to be able to control the severity of infarction and avoid mechanical damage to the vessels and the brain itself, these models are limited to the study of mechanisms of cell death, neuroprotective agents, or any other study not requiring the presence of an actual blood clot. These models have contributed significantly to stroke research; however, they are not suitable for studying the pathophysiology of cerebral ischemia infarction treated with thrombolytic or antiplatelet agents (Wang et al., 2001a). Thrombolysis of occluded arteries reduces the degree of brain injury and results in an improved clinical outcome if the therapeutic agent is delivered soon after ischemic injury (Kwiatkowski et al., 1999; Wang et al., 2001a). Therapeutic thrombolysis, therefore, holds great promise for stroke patients and has stimulated the development of various experimental animal models of stroke. Thrombolytic and antiplatelet agents cannot be studied using this model because their efficacy arises from their ability to disintegrate a blood clot and allow for reperfusion. Also, it has been observed that occlusion of the MCA by a platelet thrombus produces more serious consequences for ischemic cells than occlusion by a surgical clip, even though the occlusion was maintained for the same time period and complete reperfusion of MCA was attained afterwards (Dietrich et al., 1989). This is probably due to secondary occlusions of the downstream microvasculature by fragments generated from the clot as it is lysed spontaneously or therapeutically. These downstream occlusions of the microvasculature further exacerbate the ischemic injury.

Compared to non-thromboembolic models, cerebral focal ischemia induced by thromboembolic methods recapitulates the events that occur in humans and therefore provides a useful tool to study thrombolytic agents in ischemic brain injury (Wang et al.,

2001a; Yang et al., 2002). Therefore, for the present experiments, a rat model of MCAO required the MCA to be occluded by a clot. Besides more closely resembling the clinical situation than the models described thus far, a model such as this will allow for assessment of efficacy of thrombolytic agents to be used in present experiments. Presently, a number of these thromboembolic models exist; however, they differ in a number of factors such as, reliability, complexity of the surgical procedure, cost and time effectiveness, and the level of procedural stress to the animal.

One of these thromboembolic models involves the use of photosensitive dyes. The photochemical models work by application of a specific wavelength of light over an area of the rat's skull during intravenous infusion with a photosensitive dye. The dye reacts upon activation by the light to produce oxygen radicals which peroxidize lipids in the endothelial cell membranes, triggering thrombosis (Watson et al., 1985). When performed correctly, this method produces very consistent infarcts (Nakase et al., 1997). Unlike the other occlusion models, the time course of blood-brain barrier disruption is very different in this model from that in human cerebral ischemia (Forsting et al., 1994). Therefore the model has limited validity in focal ischemia research.

Another model that has been described involves injection of crushed human blood clot fragments (about 100μm - 300μm in length) into the internal carotid artery (Papadopoulos et al., 1987). Although this results in an actual embolic occlusion of cerebral arteries, and the MCA is very likely to be obstructed in this model, the final placement of the embolus is difficult to control. Therefore the location and size of the

infarct varies with this model.

This shortcoming was effectively tackled by the development of a model in which thrombin (blood clotting agent) was injected at the base of the MCA itself. Magnetic resonance imaging (MRI) was used to document that thrombin injection into the MCA was followed by reduced cerebral blood flow in the affected hemisphere. Administration of tissue plasminogen activator (tPA) was followed by restoration of blood flow. Post-mortem analysis of the rat brains revealed fibrin-rich clots at the base of the MCA and infarcts that were consistent in location and volume. The size of the infarct was controlled by the amount of thrombin injected (Zhang et al., 1997).

The MCAo model used in the current experiments entails injection of a preformed thrombus into the base of the MCA. The model was developed in our lab and has since been used to investigate various mechanisms of cerebral ischemia and efficacy of therapeutic treatments (Wang et al., 2001c). Not only does this model employ induction of cerebral ischemia with a preformed clot, the placement of the clot is at the base of the MCA itself. This minimizes the variation in infarct size and ensures that the same brain area is affected in all the experimental rats. The size of the injected clot (controlled by the volume of blood used to make the clot) affects the severity of infarction.

This model is advantageous over the Zhang model because the surgical procedure is simpler and less traumatic for the rats, the size of the clot is more accurately controlled, and there is no possibility of clots forming in other areas of the brain by thrombin being

carried by the blood to other regions. Therefore, the accuracy and reliability of the ischemic infarct is increased. The procedure for preparing this model is described in detail below in the section entitled "Embolic occlusion of the MCA".

Techniques for measuring infarction size

Following cerebral ischemic injury in the rat, one requires an appropriate technique to measure the infarct size, in order to quantify the resultant brain injury. There are a number of methods to determine the size of the infarct in rat brain. Any method must involve differentiating the dead tissue from the healthy brain tissue followed by some sort of quantitative analysis. Comparing mean infarct sizes of rat treatment groups is a powerful way of ranking the efficacy of the treatments tested (Hsu 1993).

One of the techniques involves the use of MRI, which allows for visualization of an infarct *in vivo*. The infarction can be viewed as it evolves over time and the brain can be viewed as cross sections in any plane. Achieving a resolution of 150μm is not uncommon (van Bruggen et al., 1999). While this is without a doubt the most technically advanced method of analyzing size of the infarct, it is also the most expensive. MRI allows for visualization of infarcts as early as 30 min after the ischemic insult (Minematsu et al., 1992) and is the perfect tool for studying changes in infarction over time. However, its high cost and its poor resolution relative to histological methods rendered it an unsuitable method for imaging infarcts for the purposes of this study.

Hematoxylin and eosin (H & E) staining is a very common histological procedure. It is used to stain many types of cells, from bone tissue to brain tissue. The resulting sections have two colors – blue due to hematoxylin and pink due to eosin. Hemotoxylin is a basic stain; thus it preferentially binds to acidic structures (e.g. nucleic acids, giving the nuclei and cytoplasmic structures containing RNA a very dark bluish black appearance). Cytoplasmic structures that do not contain acidic molecules preferentially bind eosin and appear pink. The result is a pink (light) background dotted with blue (dark) stained structures. The stark contrast between pink and blue allows for most cell structures to be seen clearly under light microscopy.

Because of the many uses for H & E staining, many different procedures exist for this type of staining, depending on the application (e.g. type of tissue being stained). H & E staining is useful for determining the extent of infarction in the brain. Dead tissue appears lighter in color than the healthy brain tissue when H & E-stained cerebral tissue sections are viewed under a light microscope (Bederson et al., 1986a). Cellular organelles (including the nuclei) rapidly disintegrate in ischemic neurons (Choi and Rotham, 1990). As mentioned earlier, the nuclei are stained by hematoxylin and appear dark blue; therefore the disintegration of nuclei in ischemic neurons results in weaker or absent hematoxylin staining of ishemic cells. It is for this reason that the healthy tissue appears darker than the infarct.

Many studies of cerebral infarction in rodents employ H & E staining to quantify the brain infarct (Alexis et al., 1995; van der Worp et al., 1998; Soonthon-Brant et al., 1999;

Tsuji et al., 2000). The method involves decapitation followed by immediate removal and instant freezing of the brain. The brain is typically frozen in liquid nitrogen or methanol or any inert liquid chilled to -20°C or colder. Freezing the brain allows for ultra-thin sectioning needed for proper staining and microscopic visualization of the tissue. A number of coronal sections, usually between 10μm and 25μm thick, are cut equidistant from each other using cryostat machinery. After these sections are stained with hematoxylin and eosin, they are viewed under a microscope or scanned at a high resolution and the infarct borders are marked on each slide. Demarcating the borders allows for the calculation of infarct area for each section by calculating the number of pixels in the infarcted area. The infarct area of a given section is multiplied by the distance between adjacent sections to yield an infarct volume. The sum of the infarct volumes (of all the sections) represents the estimated total infarct volume of the brain. A greater number of coronal sections results in a more accurate estimate of the infarct volume. This is an accurate but time-consuming method of quantifying the infarction in rodent models of cerebral ischemia.

The tetrazolium salt 2,3,5-triphenyltetrazolium chloride (TTC) is used to determine tissue death in many different tissue types, including cerebral infarcts. The colorless tetrazolium ions are reduced by dehydrogenase enzymes (e.g. succinate dehydrogenase) of the mitochondria to the pigmented formazan ions (Nachlas et al., 1957). TTC is therefore a mitochondrial stain; only those cells with functional mitochondria take up the red stain and appear red. The dead brain tissue (lacking mitochondria) appears white. The total staining procedure, including removal of the brain from the cranium followed

by slicing and staining, takes less than 30 minutes, which makes it a very convenient method of differentiation of the infarcted tissue from healthy brain tissue in coronal cross sections.

This technique is acceptable when the state of the mitochondria accurately reflects the state of the cells. If, however, functional mitochondria exist in cells whose nuclei have lysed, the cell will still stain. For this reason, TTC can only be used to accurately measure infarction in rat brains a minimum of 24 hours post occlusion (Hatfield et al., 1991).

The three methods described, TTC staining, H & E staining, and MRI imaging, are all accurate methods of measuring the infarct size. This is strongly supported by the high correlation observed between these methods when they are used to study a given sample. When compared with each other for measurement of infarct size, MRI and H & E have a correlation coefficient of 0.95 while H & E and TTC have a correlation coefficient of 0.98 (Barone et al., 1991).

Of the techniques discussed, TTC staining is the cheapest and most convenient, and its accuracy is demonstrated by the high correlation with the H & E method, which is considered to be the most accurate method. For TTC-stained sections, the infarct size can be calculated within minutes after the brain slices are immersed in the TTC solution for 20 minutes. This is in contrast to the hours required to achieve these results using H & E.

Detailed procedures for TTC staining and infarct measurement are described below in the section entitled "Measurement of infarction size".

Effects of hyperthermia on extent of ischemic injury

Numerous studies have demonstrated the deleterious effects of controlled hyperthermia in both global (Dietrich et al., 1990a; Coimbra et al., 1996) and non-thromboembolic models of focal ischemia (Chen et al., 1991). However, the effects of hyperthermia in the MCA model of cerebral ischemia have not been examined. Additionally, many clinical studies have reported body temperature to be positively correlated with severity of stroke outcome (Azzimondi et al., 1995; Reith et al., 1996; Kammersgaard et al., 2000); however, there are no data on the effects of hyperthermia on the efficacy of therapeutic thrombolysis. The aim of this first set of experiments was to examine the effects of controlled hyperthermia on the extent of ischemic injury in focal embolic model of cerebral ischemia in rats.

To carry out these experiments, a rat model of embolic occlusion of the MCA was used, the detailed procedure for which will be described shortly. However, prior to carrying out the required experiments, an appropriate method for administering hyperthermia and taking temperature measurements was required. Therefore, an initial mini-experiment setup was devised as an efficient mechanism for administering hyperthermia, while at the same time permitting active monitoring of temperature.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats, weighing 250 – 300g, were purchased from Charles River (St. Constant, Canada). The rats were housed in a 12 h light:dark cycle and had free access to water and food. Animal care and the general protocols for animal use were approved by the Animal Ethics Committee of the University of Alberta.

Administration of hyperthermia

The initial plan of the experiment required induction of hyperthermia and measuring brain temperature before, during, and for three hours after induction of ischemic injury, However; putting a temperature probe in the brain during the surgery was not feasible as probe was to be inserted from the dorsal side but the surgical procedure required the rat to be placed on the surgical station with its ventral side facing up and the dorsal side of its head in firm contact with the surgical plate. Additionally, placing a temperature sensitive probe in the brain would have confounded the calculations for infarct volume as the probe itself would have also caused injury to the brain. This problem was resolved by simultaneously measuring brain and rectal temperatures in a group of rats (n=5) and correlating the rectal temperature to the level of brain temperature, which was the official reported temperature in all publications.

A total of five animals were used in this preliminary study. Brain and rectal temperatures were recorded at two temperature points. Rats were anesthetized and the skull was exposed by making an incision on the dorsal aspect of the head. Underlying tissue was

cleared and a hole was drilled at 1.5mm anterior and 2.5mm lateral to the bregma. Brain temperatures were recorded by inserting a temperature-sensitive probe in the right striatum region with the help of a stereotactic frame. Hyperthermia was induced with a feed-back controlled heating system (Harvard Apparatus). The temperatures were raised to brain temperature of approximately 39°C and maintained for a period of three hours. Data showed that temperature in the brain was 0.5°C lower than the rectal temperature (Table 2.1).

Table 2.1: Rat brain and rectal temperatures after induction of hyperthermia

Animals	First measured point		Second measured point	
	Brain temp. (°C)	Rectal temp. (°C)	Brain temp. (°C)	Rectal temp. (°C)
1	38.12	38.65	39.04	39.53
2	38.01	38.51	39.01	39.50
3	38.22	38.71	39.00	39.48
4	37.98	38.50	38.98	39.50
5	38.00	38.49	39.04	39.51
Average difference (°C)	0.51		0.49	

Preparation of the catheter for injection of the thrombus

Injecting a thrombus into the MCA of the rat requires a modified catheter that can be inserted through the small arterial vasculature to reach the MCA. The catheter consists of two pieces of polyethylene (PE) tubing joined together. The tubing was purchased from Becton Dickinson & Co., New Jersey, USA. The (large) PE-50 tubing has an outside diameter of 0.965mm and an inside diameter of 0.58mm. It attaches to the syringe and holds the bulk of fluid (i.e. blood, thrombin or saline). The (small) PE-10 tubing has an outside diameter of 0.61mm and an inside diameter of 0.28mm. This is the tubing that is introduced into the internal carotid artery (ICA) and advanced to the MCA as will be described later. The PE-10 tubing is heated until it softens so that it can be stretched so that the outside diameter is reduced to approximately 0.25mm at one end in order to easily advance to the MCA. Tubing thicker than 0.3mm will likely become stuck in the ICA before it reaches the MCA. After thinning, approximately 20 – 22mm of the modified PE-10 tube is cut so that one tip is 0.25mm diameter and the other tip retains its original diameter. A number of these modified PE-10 catheters need to be prepared as they wear out over time through repeated manipulations while advancing them to the MCA. Before injection, the thick side of the PE-10 tube is inserted into the PE-50 tube, which contains the pre-formed blood clot. The PE-10 tube is marked at 17mm (length required to reach the MCA from the initial entry point) from its tip. Clots are formed in the PE-50 tubing and stable clot regions measuring 15mm are cut from the tubing to be used for injections (Figure 2.2).

Figure 2.2: Modified catheters for clot injection

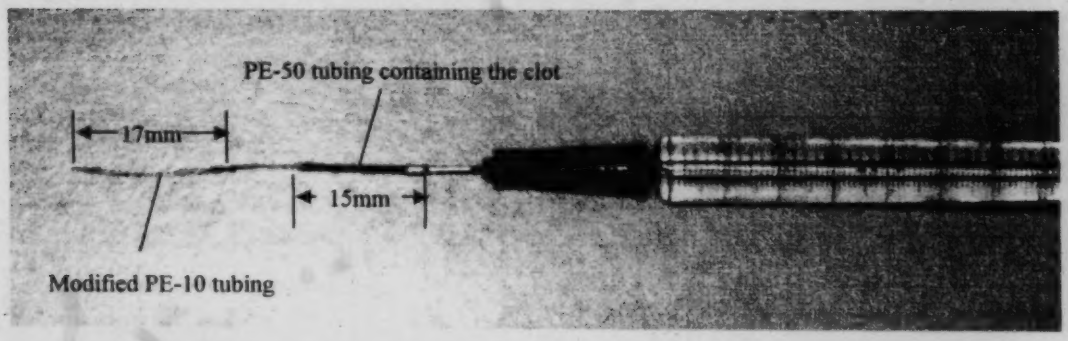

Figure 2.2: PE-50 tubing containing thrombin is inserted into the common carotid artery (CCA) to draw blood and allowed to sit at room temperature for at least 15 minutes for the clot to stabilize. The tubing is then inspected and 15mm long clot-rich sections are cut out. The 15mm long PE-50 tubing containing the clot is then attached to a Hamilton syringe (100μL) filled with saline. The modified PE-10 tubing (with a smaller diameter on the front end) is then inserted into the other end of the PE-50 tubing. The PE-10 tubing is marked at 17mm point from its tip. Only the PE-10 tubing (17mm) enters the ICA.

Embolic occlusion of the MCA

Injection of a clot in the right middle cerebral artery (rMCA) presents a challenge because the MCA cannot be accessed directly. Instead, a catheter is advanced 17mm up the right internal carotid artery (rICA), so that the tip of the catheter is in close proximity to the rMCA (within 2mm). Embolic focal cerebral ischemia is induced by embolizing a pre-formed clot into the MCA. All arteries referred to will be on the right side of the body.

The animals are anesthetized initially with 3.0% halothane (MTC pharmaceuticals, Cambridge, Canada) and then maintained with 1.5% halothane in a 70:30 nitrous oxide:oxygen mixture with a face mask during surgery on a small animal operating station with a heating system (Harvard Apparatus, Saint Laurent, Canada). In addition to this, the breathing rate and depth are used to determine the correct halothane setting (e.g. if breathing becomes shallow, the halothane setting is lowered).

A longitudinal incision of 1.5 cm in length is made in the midline of the ventral cervical skin at the level of the neck and the CCA, external carotid artery (ECA), and ICA are exposed by removing as much soft tissue from these arteries as possible while avoiding unnecessary damage.

The CCA divides into the ECA and ICA (Figure 2.3). The arteries are exposed at the point of bifurcation. As mentioned, a catheter will be advanced up the ICA, but the point of entry will be the ECA, not the ICA. An incision in a large carotid artery cannot be closed, and the only way to prevent bleeding after the surgery is to seal the artery at both

sides of the incision. Blood flow through the ICA enters the brain and the MCA, so tying this artery would be lethal to the rat. On the other hand, the ECA does not irrigate the brain and therefore ligating the ECA will not compromise blood flow to the brain.

The fact that the ICA and ECA both branch off in the same direction presents an obstacle because a catheter introduced into the ECA will traverse down the CCA and not up the ICA as desired (Figure 2.3). To circumvent this problem, the ECA has to be realigned in such a way so that insertion of a catheter in the ECA would result in its travel up the ICA and not down the CCA. However, before realigning the ECA, a minor artery branching off from ECA has to be electro-cauterized using a bipolar electrosurgical unit (Vetroson®, Summit Hill Laboratories, Navesink, NJ, USA); otherwise this artery will break once the ECA is realigned. Following this, the distal portion of the ECA is ligated with two sutures and the ECA is cut between these two sutures (Figure 2.4). The junction where the ECA and ICA meet is cleaned by removal of excess tissue and electro-cauterization of another minor artery branching from the proximal region of ECA. Then the ~6mm of ECA still attached to the junction is pulled back 180° to bring it in a straight line with the ICA so that insertion of a catheter in the ECA would result in its travel up the ICA and not down the CCA (Figure 2.5). A hole must be made in the ECA to allow for entry of the catheter. However, making an incision in the ECA at this point would result in heavy bleeding. To prevent this, the CCA and ICA are temporarily clamped using microvascular clips (FST, North Vancouver, Canada). If only the CCA is clamped, blood can travel from the left to the right side of the circulation at a number of points downstream where the two circulations meet and travel retrograde down the ICA and

bleed out of the incision in the ECA. That is why the ICA must also be clamped. Before making an incision in the ECA, the portion of the ECA distal to the incision (i.e. closer to the junction) is surrounded by a loosely tied silk thread such that tugging on the thread would press the walls of the ECA on the catheter inside it (Figure 2.5). After the CCA and ICA are clamped with microvascular clamps and a loose knot is tied around the ECA, a small incision is made on the wall of the ECA with a pair of spring scissors.

Figure 2.3: Carotid arteries of the neck

Figure 2.3: The CCA divides into the ECA and ICA. At first, only the ECA and CCA are visible. The ICA is exposed after removing the soft tissue at the bifurcation of the CCA to the ECA and ICA. If viewed from directly above, the ICA is not visible as it is hidden underneath the ECA.

Figure 2.4: Ligation of ECA before it is cut

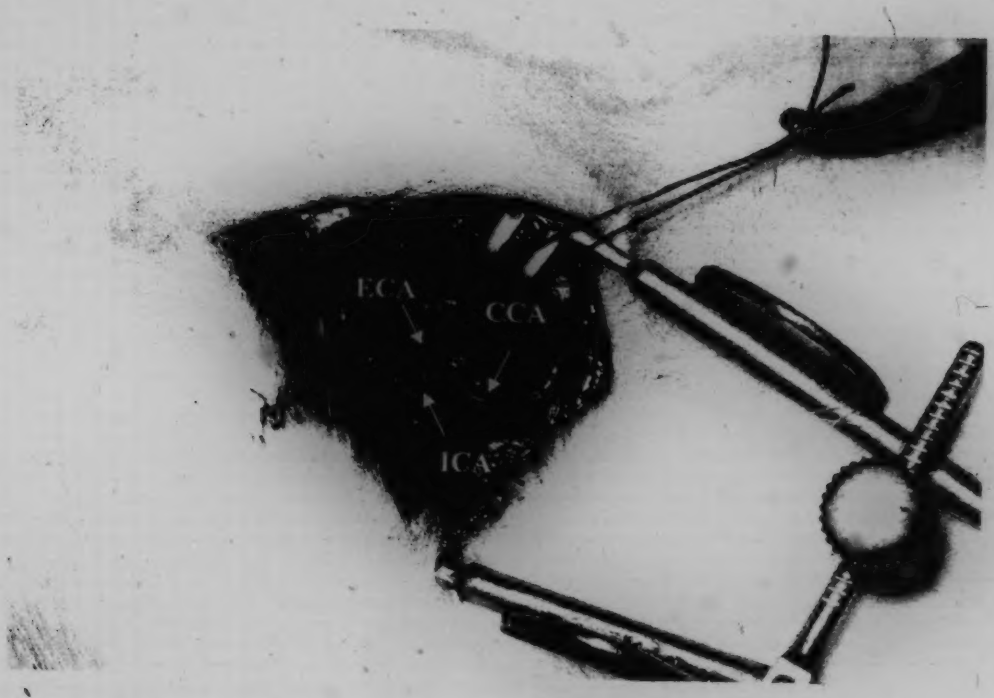

Figure 2.4: The distal portion of ECA is ligated with two sutures before it is cut in between them. For one of the sutures, a longer length of the thread is maintained and held with a clamp in order to pull back and realign the section of the ECA.

Figure 2.5: Aligning the ECA with the ICA

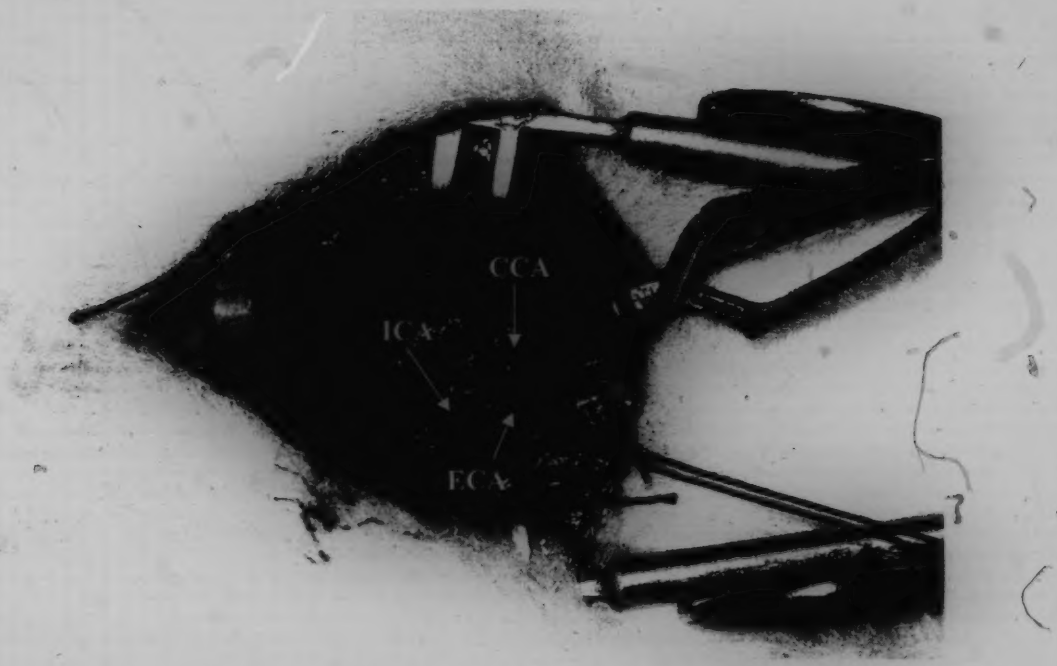

Figure 2.5: After cutting between the two sutures, the ECA is pulled back 180° in order to align it with the ICA so that insertion of a catheter in the ECA would result in its travel up the ICA and not down the CCA. Before making a puncture in the ECA to insert the catheter, both the ICA and CCA are clamped and a loose silk thread is tied around the ECA. Once the catheter is inserted into the ECA and advanced to the ICA, the ICA clamp is removed and the loose silk thread is lightly tightened on the ECA to prevent the catheter from sliding back out.

Preparation of embolus and injection

The clot formation technique is modified slightly from the standard procedure technique used in our lab (Wang et al., 2001a) to simplify the clot forming procedure and to cut down on the time required for each surgical procedure. In brief, a 30mm long Polyethylene (PE-10) catheter (Becton-Dikinson, NJ, USA) connected to a PE-50 tubing (120mm in length) filled with bovine thrombin (10 NIH U/μl) is introduced into the lumen of the right ECA via the puncture and advanced to the junction. Removal of the clamp on the CCA or the ICA at this point will cause blood to flow around the catheter and out the incision, so the silk thread on the ECA (Figure 2.5) is tugged gently. The resulting knot presses the walls of the ECA against the catheter, preventing blood from flowing around it. This knot must not be too tight or the catheter will not be able to slide through the ECA.

At this point, the clamp on the CCA is removed to collect blood. The catheter is then removed and the CCA clamped again. The PE-50 tubing is kept undisturbed at room temperature for 15 min to allow for clot formation. Once the clot is formed, the tubing is inspected for clot-rich portions and 15mm pieces of tubing, containing stable clots, are cut out. A 15mm clot-containing piece of PE-50 tubing is attached to a modified PE-10 catheter on one side and a 100-μl Hamilton syringe (Fisher) on the other side (Figure 2.2). For the sham animals, a 15mm piece of PE-50 tubing filled with normal saline is used.

The catheter is introduced into the ECA and the clamp on the ICA is removed to allow the catheter to advance 17mm up the ICA until its tip is 1 – 2mm away from the origin of the MCA. The clot is then gently injected. The CCA remains clamped and the catheter remains inserted for five minutes after clot injection to allow the clot to settle in the MCA. The catheter blocks some collateral blood flow (Figure 2.6), preventing the clot from washing away from the MCA. The retrograde blood pressure in the ICA attempts to force the clot backward. The catheter blocks it, leaving the blood and the clot with only one way to travel, up the MCA (Figure 2.6). After this delay, the catheter is removed and the ECA is ligated by tightening the suture around the origin of the ECA to prevent bleeding from the incision. The clamp on the CCA is removed and the surgical wound closed with skin staples. For the normothermic animals, brain temperature is maintained at 37°C and for the hyperthermic animals, it is maintained at 39°C before embolizing a clot into the MCA, throughout the surgery, and for 3 h following ischemic injury by a feedback controlled heating system.

Figure 2.6: Embolic model of MCA occlusion

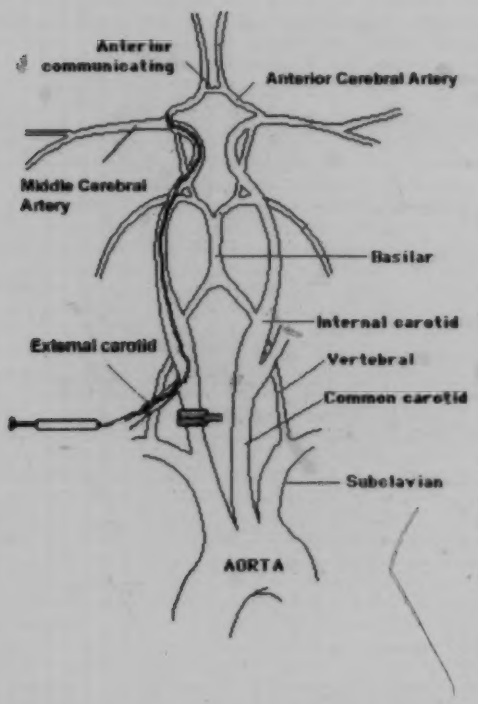

Figure 2.6: In the embolic model of MCA occlusion, the right CCA is clamped and the catheter is introduced into the right ECA and advanced up the right ICA, to within 1 – 2mm of the right MCA. The pre-formed clot is gently injected and the catheter is kept in place to allow for the clot to move into the MCA. Blood from the left side of the circulation can traverse through the anterior communicating artery, down the anterior cerebral artery and into the MCA (and carry the clot with it), but not down the ICA because the catheter blocks that route. Upon MCAo, blood from the left side of the circulation exerts a force on the clot, pushing it firmly into the MCA.

Measurement of neurological deficits and seizure activity

Neurological deficits and seizure activity were measured at 24 hours following ischemic injury. Neurological deficits were determined using a modified Bederson's scoring system (Bèderson et al., 1986b) as described. 0: no observable deficit; 1: forelimb flexion; 2: forelimb flexion plus decreased resistance to lateral push; 3: unidirectional circling; 4: unidirectional circling plus decreased level of consciousness. Seizure activity was classified with Racine scores (Racine, 1972). 0: no seizure observed; 1: rhythmic mouth and facial movements; 2: rhythmic head nodding; 3: forelimb clonus; 4: rearing and bilateral forelimb clonus; 5: rearing and falling.

Measurement of infarction size

The procedures for assessment of infarction volume have been reported previously in detail by our lab (Yang et al., 1998). In brief, at the end of each experiment (48 h after embolization), the animal was sacrificed and the brain was removed and sectioned coronally at a thickness of 2mm per section to generate eight sections. The sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) solution (2% w/v) (Sigma-Aldrich, Oakville, Canada) at 37°C in an incubator (Isotemp Incubator, Fischer Scientific) for 20 minutes. The infracted region failed to stain and appeared pale white in comparison to the dark red stained non-injured regions. The slices were then scanned using a flatbed color scanner (Scanjet 4p, Hewlett-Packard) and the images were analyzed using an image-processing software program Adobe Photoshop, version 6.0. Brain infarct size was measured manually by outlining the margins of infarct areas to reveal the total number of pixels (area) in the infarcted region. The infarction volume

was determined by integration of the areas from the eight sections. The infarction volume was expressed as a percentage of the total volume from the ipsilateral hemisphere.

Experimental design

The present study was designed to examine the effects of hyperthermia on the extent of ischemic injury in a focal embolic model of stroke in rats. Neurological deficits and seizure activity changes with hyperthermia were also recorded. Finally, mortality in each group was also recorded. Mortality was recorded if it occurred during the hyperthermic treatment following embolization or at any time after that. Due to premature mortality and technical errors, a total of 24 rats were included in this study in order to obtain a total of 8 animals in each group for assessment of infarction volume. Two of the rats did not show any evidence of ischemic injury and were therefore excluded from the results.

Hyperthermia (n=14) was induced with a feedback controlled heating system (Harvard Apparatus). Brain temperature was raised to 39°C (rectal temperature 39.5°C) in the anesthetized rat before embolizing a clot into the MCA, and the brain temperature was maintained at this level for a period of 3 h after the embolization. In the control group (n=8), rectal temperature was maintained at 37°C before embolizing a clot into the MCA, and the brain temperature was maintained at this level for a period of 3 h after the embolization. As mentioned earlier, in a preliminary study, brain and rectal temperatures were monitored for a period of 3 h in five non-operated rats. Data showed that temperature in the brain was 0.5°C lower than in the rectal temperature. Since the

insertion of a temperature probe resulted in a physical injury to the brain which would confound our infarct volume measurements, we therefore elected to use rectal temperature and correlated it to resultant brain temperature. The data from infarct volume and behavioral tests were available from eight rats in the hyperthermia group for statistical analysis due to premature death of some rats.

Statistical analysis

The differences in infarct volumes between the two groups were analyzed with a two-tailed t-test. Neurological deficits and seizure activity were analyzed with the Mann-Whitney test and mortality was analyzed with a χ^2 test.

Results

Infarction volume

Embolizing a pre-formed clot resulted in an infarction in the territory irrigated by the MCA, mainly located in the cerebral cortex and the striatum (Figure 2.7). Animals in the normothermic group exhibited an average infarct volume of 31.9 ± 1.4% (mean ± SEM) of the ipsilateral hemisphere. In the hyperthermic animals, the infarct volume was significantly increased to 61.5 ± 2.1% ($P<0.001$, 2 tailed t-test) of the ipsilateral hemisphere, which corresponded to an increase of approximately 93% (Figure 2.8).

Figure 2.7: TTC stained sections following cerebral ischemia

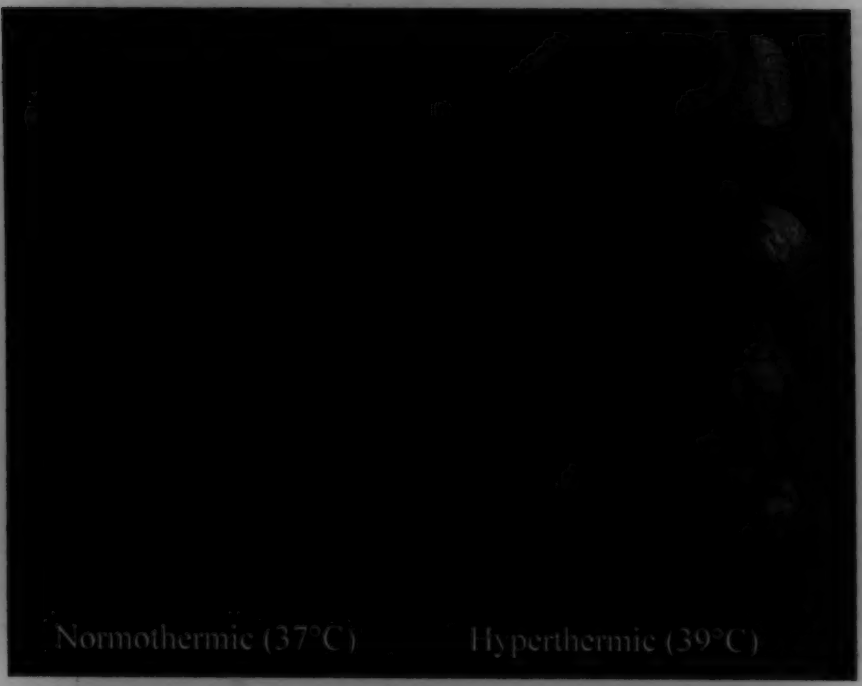

Figure 2.7: TTC stained sections of rat brains following embolization of a clot in the MCA. Dead tissue fails to stain and appears as pale white in comparison to the red stained non-injured regions. In hyperthermic brain sections, the size of infracted tissue is significantly larger as compared to the normothermic brain sections.

Figure 2.8: Effect of hyperthermia on infarction volume following ischemic brain injury

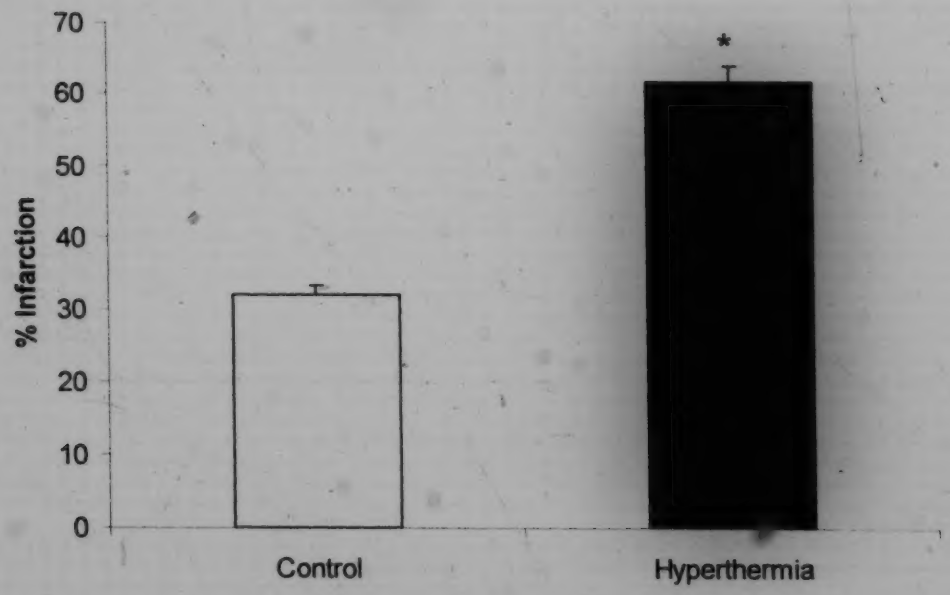

Figure 2.8: Infarct volume in the ipsilateral hemisphere following embolization of a preformed clot into the MCA. The infarct volume was measured in the TTC-stained brain sections at 48 h after embolization. Each bar represents mean ± SEM (n = 8). The infarction volume in the rats that received treatment with hyperthemia was significantly larger than in the normothermic (control) rats ($P<0.001$). * denotes significantly larger than control group.

Neurological deficits and seizure activity

Neurological deficits and seizure activity were recorded at 24 h following ischemic injury. The neurological deficit score was 2 ± 0.4 in the normothermic group and 4 ± 0.2 in the hyperthermia group. The scores of neurological deficits (Figure 2.9) were significantly higher in the hyperthermic rats than in the normothermic rats ($P<0.001$, Mann-Whitney test). Scores of seizure activity (Figure 2.10) in the normothermic and hyperthermia groups were 1 ± 0.4 and 2 ± 0.7 respectively. The seizure scores were not statistically different between these two groups ($P=0.078$, Mann-Whitney test).

Mortality

In the normothermic group, all rats survived until the end of the experiment. Six rats died prematurely following ischemic injury in the hyperthermia group (four died within 3 h; two within 24 h). The mortality in these two groups was significantly different ($P<0.05$, χ^2 test).

The present study demonstrates that treatment with controlled hyperthermia significantly increased neuronal damage following ischemic injury in an embolic model of stroke in rats. Treatment with hyperthermia (brain temperature of 39°C) during and for 3 h following embolic occlusion of the MCA significantly increased infarct volume and neurological deficits. Moreover, hyperthermia also caused an increase in mortality.

Figure 2.9: Effect of hyperthermia on neurological deficits following cerebral ischemic injury

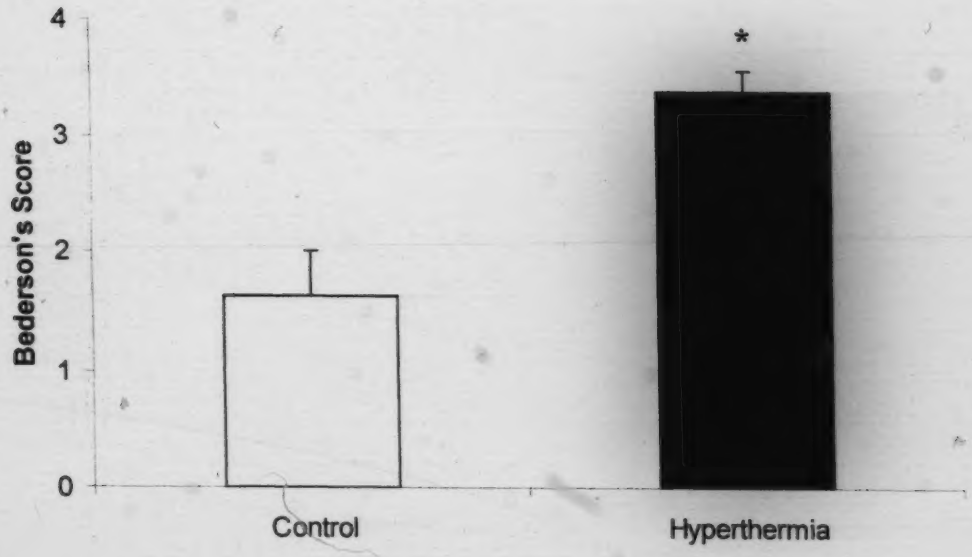

Figure 2.9: The neurological deficit scores following ischemic brain injury. Neurological deficits were recorded at 24 h after embolizing a preformed clot into the MCA. Each bar represents mean ± SEM (n = 8). The scores in the rats that received treatment with hyperthemia were significantly higher than in the normothermic rats ($P<0.001$). * denotes significantly higher than control group.

Figure 2.10: Effect of hyperthermia on seizure activity following cerebral ischemic injury

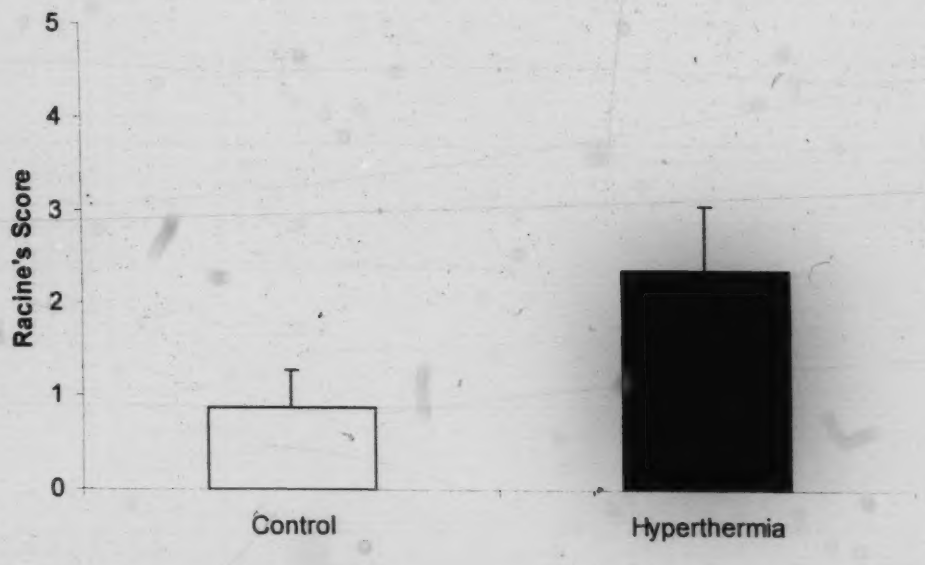

Figure 2.10: The seizure activity scores following ischemic brain injury. Scores for seizure activity were recorded at 24 h after embolizing a preformed clot into the MCA. Each bar represents mean ± SEM (n = 8). There was no significant difference in seizure activity scores between the two groups, although they tended to be increased in hyperthermic rats.

Hyperthermia has been shown to increase infarct size in global ischemic brain injury (Dietrich et al., 1990a; Coimbra et al., 1996). Hyperthermia also worsens the ischemic injury in a reperfusion injury model induced by blockage of the MCA with a suture (Chen et al., 1991). However, the results of hyperthermia in permanent MCA occlusion are still controversial. For example, one study showed that infarction resulting from permanent MCA occlusion was not affected by hyperthermia (Morikawa et al., 1992). Other investigators, however, using a rat model of permanent MCA and ipsilateral carotid artery occlusion, showed an increase in the volume of infarction (Chen et al., 1991). Results from the present study clearly show that hyperthermia is detrimental for the ischemic injured brain in the embolic model of stroke.

Hyperthermia may act through several mechanisms to worsen the injury due to cerebral ischemia. One of these mechanisms is the effect on release of excitatory neurotransmitters. The release of neurotransmitters in both global and focal ischemia is elevated by hyperthermia and diminished by hypothermia. Glutamate levels in the forebrain following ischemic injury are increased by 37-fold and tend to persist longer during the recirculation period in hyperthermic rats (Sternau et al., 1992). Another study reported that in normothermic ischemic animals, the excitotoxic index, a composite measure of neurotransmitter release, is increased by only 2-fold where as in the hyperthermic ischemic rats, the same index showed a 20-fold elevation (Globus et al., 1991). This implies that under hyperthermic conditions, there may exist a potential for greatly enhanced excitotoxicity. Another mechanism through which hyperthermia may work to augment the damage following cerebral ischemia is oxygen radical production.

Cortical oxygen radical production during the early recirculation period following an ischemic insult is greatly influenced by intraischemic brain temperature. Oxygen radical production is increased 4- to 5-fold following hyperthermic ischemia; in contrast, only 2- to 3-fold elevation is seen following normothermic ischemia (Globus et al., 1995). Blood-brain barrier changes are also markedly exacerbated following hyperthermia in ischemic animals. The mild extravasation of protein tracers across the barrier under normothermic ischemia is markedly exaggerated by mild intraischemic hyperthermia (Dietrich et al., 1990b). Taken together, the above evidence indicates that hyperthermia has deleterious effects on ischemic injury in the brain.

In summary, the present study shows for the first time, in an embolic model of cerebral ischemia induced by injection of preformed clots into the MCA, that hyperthermia significantly increases the volume of brain infarction following ischemic insult. This is accompanied with an adverse behavioral profile as well an increased mortality rate.

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Noor R, Wang C, Shuaib A. Effects of hyperthermia on infarct volume in embolic model of cerebral ischemia. Neurosci Lett. 2003 Oct 2;349(2):130-2.

Chapter 3: Hyperthermia and thrombolysis

Thromboembolism is involved in 80 – 90% of stroke cases (Albers 2001) and the majority of ischemic episodes occur as a result of occlusion of the MCA or its branches (Wang et al., 2001a). After an ischemic event, neuronal death evolves in a time-dependent fashion determined by both the duration and severity of blood flow interruption (Siesjo 1992). Delayed reperfusion can lead to negative sequelae such as the breakdown of BBB, and the development of hemorrhagic transformation and edema (Aoki et al., 2002). As the majority of ischemic strokes are caused by thrombotic or thromboembolic arterial occlusions, therapeutic strategies designed to restore cerebral perfusion hold great promise for these patients (del Zoppo et al., 1992). Indeed, intravenous infusion of tPA is the only scientifically proven effective therapy for acute ischemic stroke (Albers 2001). There is increasing evidence that moderate hyperthermia, when present during or after a period of brain ischemia or trauma, markedly exacerbates the degree of resulting neural injury (Ginsberg and Busto 1998). Clinical studies also indicated that 50% of all stroke patients have escalated body temperatures within 48 h of a stroke insult (Azzimondi et al., 1995). In a prospective study, body temperature proved to be highly correlated with initial stroke severity, infarct size, mortality, and poor outcome (Reith et al., 1996).

In animal models of transient forebrain ischemia, elevations of intra-ischemic brain temperature were reported to enhance and accelerate ischemic injury in vulnerable brain regions and induce damage to structures not ordinarily affected (Dietrich et al., 1990a). Other studies in focal cerebral ischemia have shown that hyperthermia is deleterious in

focal ischemia and that the effects of otherwise neuroprotective drugs in ischemia may be nullified or become deleterious due to hyperthermia (Ginsberg and Busto 1998).

As discussed in Chapter 2 above, we have shown previously that hyperthermia significantly increased neuronal damage following ischemic injury in a focal embolic model of stroke in rats. Hyperthermia during and for 3 h following embolic occlusion of the MCA significantly increased infarct volume and neurological deficits (Noor et al., 2003). Numerous other studies have also demonstrated the deleterious effects of controlled hyperthermia in both global (Coimbra et al., 1996; Dietrich et al., 1990a) and non-thromboembolic models of focal ischemia (Chen et al., 1991).

There is a paucity of data on the effects of hyperthermia on the efficacy of therapeutic thrombolysis. Since thrombolysis with tPA is the first line of treatment for acute stroke in the clinical setting, knowledge about influence of body temperature on efficacy of thrombolytic therapy in embolic stroke is of paramount importance. The aim of the present study was to examine the effects of controlled hyperthermia on the efficacy of thrombolytic therapy following ischemic injury in focal embolic model of cerebral ischemia in rats.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats, weighing 250 – 300g, were purchased from Charles River (St. Constant, Canada). The rats were housed in a 12 h light:dark cycle and had

free access to water and food. Animal care and the general protocols for animal use were approved by the Animal Ethics Committee of the University of Alberta.

Administration of hyperthermia

Hyperthermia was administered as described previously in Chapter 2. In this study, however, another intermediate hyperthermia group was added to show the dose-dependent effect of temperature on cerebral ischemic injury. For the normothermic animals, brain temperature was maintained at 37°C and for the hyperthermic animals, it was maintained at 38°C or 39°C before embolizing a clot into the MCA, throughout the surgery, and for 3 h following ischemic insult.

Embolic occlusion of the MCA

The procedures for embolic occlusion of the MCA were described in detail in Chapter 2. Briefly, embolic focal cerebral ischemia was induced by embolizing a pre-formed clot into the MCA. Following embolization, saline or tPA (10mg/kg) was infused intravenously via the tail vein at 30 min post MCA occlusion.

Intravenous infusions via the tail vein

Following embolic occlusion of the MCA, rats are kept anesthetized with 1.5% halothane in a 70:30 nitrous oxide to oxygen air mixture while undergoing temperature treatments for 3 hours. For the present experiments, tPA or saline administration was started at 30 min post MCA occlusion. The tail vein was chosen as the route of administration because of a smaller risk of bleeding as compared to the jugular vein route. Also,

following drug infusions, the utilized vein has to be ligated to prevent continued bleeding. Ligating the jugular vein on one side could have increased blood pressure in the brain, complicating evolution of the injury. The surgical procedure described below using the tail vein is also less traumatic for the animal as compared to the surgical procedure for drug administration via the jugular vein.

In order to access the rat tail vein, an incision (1.5 cm to 2 cm) is made distally along the ventral aspect of the tail. Due to collateral circulation, an incision can only be made by clamping the vein at two points and puncturing a small hole in between those points. A catheter made of PE-50 tubing is attached to a syringe containing the drug and then inserted into the tail vein with the tip of the catheter facing the direction of the blood flow. The syringe is attached to an infusion pump that can be programmed to infuse the drug at a given rate and time period. One clamp is removed to allow the catheter to advance deeper into the vein. A silk thread is tied around the vein-wall so that it is snug against the catheter, preventing back-flow of drug and/or blood past the catheter.

The part of the vein held by the other clamp is tied shut (with silk thread) and the clamp is removed. After the infusion is completed, the catheter is removed from the vein and the thread that was used to hold the catheter in place is tightened to seal that part of the vein also. The wound is closed with silk sutures.

Saline or tPA (10mg/kg) infusion was initiated at 30 min after the clot injection, with 1/3 of the dose given as a bolus while the remaining 2/3 was infused over 30 min.

Measurement of neurological deficits and seizure activity

Neurological deficits and seizure activity were measured at 24 hours following ischemic injury. Neurological deficits were determined using a modified Bederson's scoring system (Bederson et al., 1986b) as described above. Seizure activity was classified with Racine scores (Racine 1972) as described above.

Measurement of infarction size

The procedures for assessment of infarction volume are described in detail in Chapter 2. For the present experiments, the endpoint was set as 24 h after embolization. At the end of each experiment, the animal was sacrificed and the brain was removed and sectioned coronally at a thickness of 2mm per section to generate eight sections which were stained at 37°C for 20 minutes. The infarction volume was determined by integration of the infarcted areas from the eight sections. The infarction volume was expressed as a percentage of the total volume from the ipsilateral hemisphere.

Measurement of brain edema

In the present study, brain edema was also assessed in order to determine whether hyperthermia can influence the evolution of ischemic injury by causing brain edema which may compress the surrounding vasculature resulting in further injury. Brain edema in tPA-treated animals was also assessed to determine whether any improvements

seen in infarction volumes with tPA also translated in improvements in brain edema. Although tPA is not an anti-edema drug, it is conceivable that changes in levels of infarcted tissue could also affect the levels of associated brain edema. Brain edema was determined by calculating the volume difference between the two hemispheres and dividing by the volume of the left hemisphere (Shuaib et al., 2002).

Experimental design

The present experiments were designed to examine the effects of hyperthermia on neuroprotective efficacy of tPA following ischemic injury in a focal embolic model of stroke in rats. Neurological deficits and seizure activity changes with hyperthermia and with or without tPA treatment were also recorded. Finally, mortality in each group was also recorded. Mortality was recorded if it occurred during the hyperthermic treatment following embolization or at any time after that. If mortality occurred prior to or at the moment of embolization or if no sign of ischemic injury was seen in stained slices, those animals were designated as technical errors and excluded from the results.

Animals were randomly assigned to the following groups: normothermic (37°C) + saline (n=8), normothermic + tPA (n=8), 38°C hyperthermia + saline (n=8), 38°C hyperthermia + tPA (n=8), 39°C hyperthermia + saline (n=8), and 39°C hyperthermia + tPA (n=8). The animals were euthanized at 24 h after embolization. Because of premature mortality and technical errors, a total of 76 rats were used in order to obtain 8 animals in each group for assessment of infarct volume and edema. Six of the rats did not show any

evidence of ischemic injury and three of the rats died at the time of embolization and therefore they were excluded from the results.

Statistical analysis

The differences in infarct volume and edema were analyzed with one-way ANOVA followed by the Holm-Sidak method. Neurological deficit and seizure scores were reported as medians and interquartile ranges (25^{th} to 75^{th} percentiles). Neurological deficit and seizure scores were analyzed with the Kruskal-Wallis test. Mortality rates were compared with the χ^2 test. Differences were considered significant when $P<0.05$.

Results

Infarction volume

Embolizing a pre-formed clot resulted in an infarction in the territory irrigated by the MCA. At 24 h after the MCA occlusion, average infarct volume in the normothermic group was 35.6 ± 2.8% (mean ± SEM) of the ipsilateral hemisphere. In the hyperthermic animals, the infarct volume was significantly increased to 50.9 ± 3.7% ($P<0.001$) in the 38°C group and to 62.4 ± 2.7% ($P<0.001$) in the 39°C group (Figure 3.2). Infarct volume in the 39°C group was also significantly larger than that in the 38°C group ($P<0.001$). Following tPA treatment (Figure 3.1), the infarct volume in the normothermic and 38°C groups was significantly decreased to 15.7 ± 2.6% and 36.2 ± 3.7% ($P<0.001$), but not in the 39°C group (Figure 3.2).

Figure 3.1: TTC stained sections following ischemic injury and tPA treatment

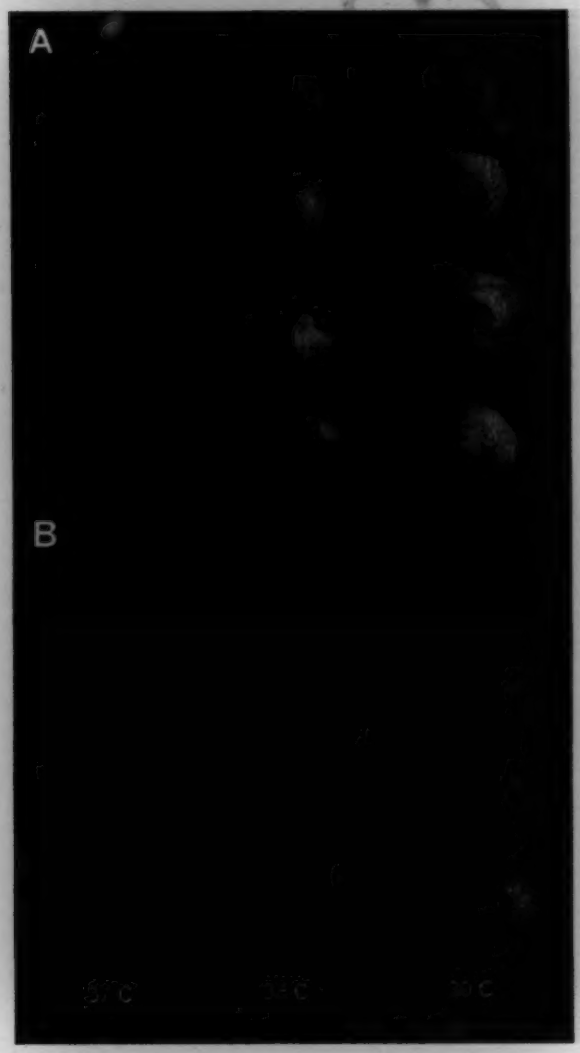

Figure 3.1: Representative TTC-stained sections of rat brains following embolization of a clot in the MCA with (B) or without (A) tPA treatment. Treatment with tPA was effective in decreasing the size of infarcted tissue in the 37°C and 38°C rats but failed to show much improvement in the 39°C rats.

Figure 3.2: Effect of hyperthermia on infarction volume following ischemic brain injury

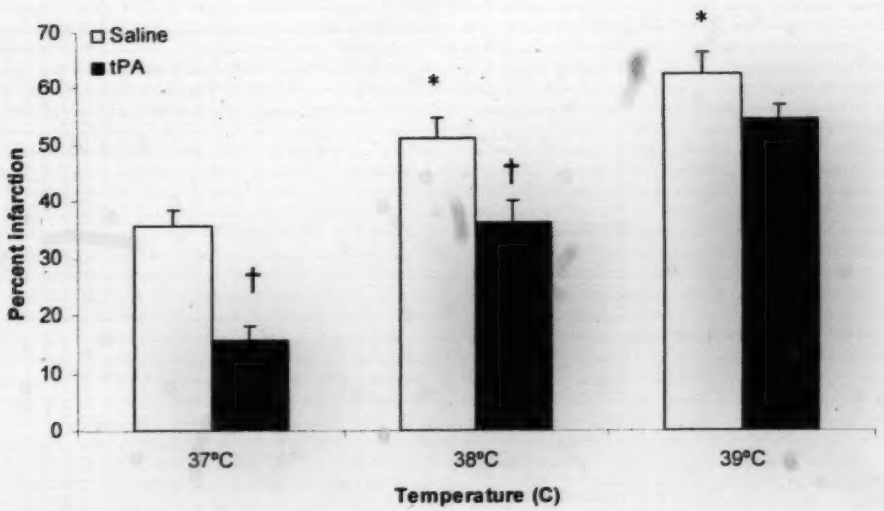

Figure 3.2: Infarct volume in the ipsilateral hemisphere following embolizing a pre-formed clot into the MCA. The infarct volume was measured in the TTC-stained brain sections at 24 h after embolization. Each bar represents mean ± SEM (n=8). * denotes significantly larger than in the normothermic rats ($P<0.001$). † denotes significantly smaller than in non-tPA-treated rats in the normothermic and the 38°C hyperthermia groups ($P<0.001$), but not in the 39°C hyperthermia group ($P=0.08$).

Brain edema

Normothermic animals exhibited an average brain edema of 10.7 ± 2.2% whereas in the 38°C and 39°C groups, brain edema was increased to 19.2 ± 1.9% and 17.8 ± 2.0% respectively (Figure 2). Brain edema was significantly increased in the 38°C group only as compared to the normothermic group ($P<0.05$).

Neurological deficits and seizure activity

Neurological deficit scores are shown in Table 3.1. The scores of neurological deficits were significantly higher in both 38°C and 39°C hyperthermia groups when compared to the normothermic animals ($P<0.05$). Treatment with tPA improved neurological deficits in the 38°C hyperthermia group only ($P<0.05$).

Seizure activity scores are shown in Table 3.2. The seizure scores were significantly increased in the 38°C hyperthermia group only ($P<0.05$). Treatment with tPA resulted in decreased seizure activity in the 38°C hyperthermia group only ($P<0.05$).

Mortality

In both normothermic groups, all rats survived until the end of the experiment. Five rats died prematurely in the saline-treated 38°C group and five rats died prematurely in the tPA-treated 38°C group. In the saline-treated 39°C group, three rats died prematurely, and five rats died prematurely in the tPA-treated 39°C group. Hyperthermia significantly increased mortality in both the 38°C and 39°C groups as compared to controls ($P<0.05$).

Figure 3.3: Effect of hyperthermia on brain edema following ischemic brain injury

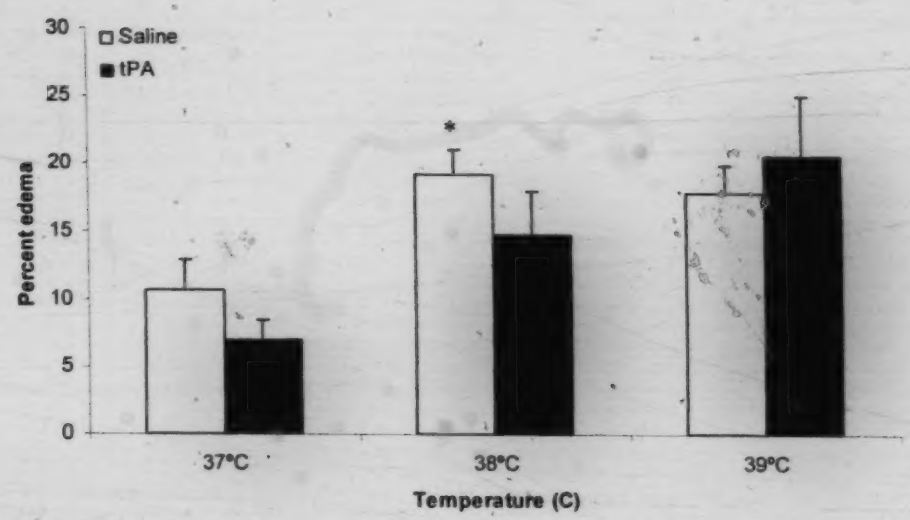

Figure 3.3: Brain edema in the ipsilateral hemisphere following embolizing a pre-formed clot into the MCA. Brain edema was measured in the TTC-stained brain sections at 24 h after embolization. Each bar represents mean ± SEM (n=8). Brain edema in the 38°C group was significantly higher ($P<0.05$) than the normothermic group, but there were no significant differences between the normothermic and 39°C group ($P=0.07$). tPA treatment did not result in any significant decrease in brain edema, compared to the non-tPA-treated groups ($P>0.05$).

Table 3.1: Neurological deficits following focal cerebral ischemia [a]

	Groups (n = 8 per group)		
	Normothermic (37°C)	Hyperthermic (38°C)	Hyperthermic (39°C)
Saline	2.5 (1.5-3)	3 (3-4)*	3.5 (3-4)*
tPA	2.5 (1-3)	2.5 (2-3)†	4 (3-4)

[a] The neurological deficits were recorded at 24 h after embolizing a pre-formed clot into the MCA. Neurological deficit scores are expressed as medians and interquartile ranges; 25th to 75th percentiles are shown in parentheses. * denotes significantly higher when compared to the normothermic animals ($P<0.05$). † denotes tPA treatment significantly improved neurological deficits when compared to the saline group ($P<0.05$).

Table 3.2: Seizure activity following focal cerebral ischemia [a]

	Groups (n=8 per group)		
	Normothermic (37°C)	Hyperthermic (38°C)	Hyperthermic (39°C)
Saline	1 (0.5-2.5)	4 (2.5-5)*	1.5 (1-4.5)
tPA	0 (0-1)	0.5 (0-1.5)†	2 (1-2)

[a] Seizure activity scores were recorded at 24 h after embolizing a pre-formed clot into the MCA. Seizure activity scores are expressed as medians and interquartile ranges; 25th to 75th percentiles are shown in parentheses. * denotes significantly higher when compared to normothermic animals ($P<0.05$). † denotes significantly lower when compared to the saline group ($P<0.05$).

As discussed in Chapter 2, our previous study examining the effects of hyperthermia on cerebral injury in an embolic model of stroke revealed that hyperthermia significantly worsened the outcome in an embolic model of stroke (Noor et al., 2003). In the present study, we examined the efficacy of thrombolytic therapy with tPA in this model under hyperthermic conditions. The present results confirmed our previous findings that hyperthermia markedly exacerbates neuronal damage following focal ischemic injury. Moreover, our data also show that hyperthermia masks the neuroprotective effects of tPA, as treatment with tPA significantly reduced infarct volume in normothermic rats whereas in moderately hyperthermic rats (38°C) the neuroprotective effects were less pronounced, and no improvement was seen in severely hyperthermic rats (39°C). tPA treatment also did not improve functional recovery, measured with behavioral tests, in the hyperthermic rats. In addition, tPA treatment also failed to decrease mortality in the hyperthermic rats. These data thus clearly show that thrombolytic therapy with tPA is not effective in ischemic brain injury in the presence of hyperthermia.

*Sections of this chapter have been published as:

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Chapter 4: Hyperthermia and vasculature

Hyperthermia may act through several mechanisms to worsen the extent of ischemic injury, details of which have been discussed in Chapter 1. In the present experiments, we used our model of focal embolic stroke in rats and examined the effects of hyperthermia on brain vasculature to determine whether the observed detrimental effects of hyperthermia on ischemic injury, as discussed above, can be explained by any changes in vascular permeability and brain reperfusion. In particular, we examined the effects of hyperthermia on BBB permeability and perfusion deficits following focal ischemia to determine whether hyperthermia plays a role in ischemic damage by affecting vascular permeability and reperfusion following cerebral ischemia. Moreover, we also carried out an *in vitro* study examining the effects of hyperthermia on fibrinolytic activity of tPA to determine whether hyperthermia affects ischemic damage by changing the enzymatic activity of tPA.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats, weighing 250 – 300g, were purchased from Charles River (St. Constant, Canada). Animal care and the general protocols for animal use were approved by the Animal Ethics Committee of the University of Alberta.

Administration of hyperthermia and embolic occlusion of the MCA

Hyperthermia was administered as described previously in Chapter 2. For the normothermic animals, brain temperature was maintained at 37°C and for the

hyperthermic animals, it was maintained at 39°C before embolizing a clot into the MCA, throughout the surgery, and for 3 h following ischemia. The procedures for embolic occlusion of the MCA were described in detail in Chapter 2. Briefly, embolic focal cerebral ischemia was induced by embolizing a pre-formed clot into the MCA.

Detection of perfusion deficits

Perfusion deficits in the ischemic injured brains were measured in order to determine whether hyperthermia has any adverse effects on reperfusion following ischemic injury. Perfusion deficits were measured at 3 h and 6 h after ischemic injury in both normothermic and hyperthermic rats.

In anesthetized animals, the right jugular vein is isolated and clamped at two points. A small puncture hole is made between the two clamped points and a PE-50 catheter, attached to a syringe filled with Evans blue, is inserted into the jugular vein towards the direction of blood flow. Evans blue (E-2129, Sigma, Oakville, ON, Canada; 2% solution in saline, 0.2 mL/100 g body weight) is injected into the right jugular vein and allowed to circulate for 10 seconds. The animals are then decapitated and their brains removed. The brains are frozen in 2-methylbutane kept on dry ice, embedded in optimal cutting temperature (OCT) medium, and stored at -70°C until analysis. For analysis, the brains are sectioned at 10μm in thickness with a cryomicrotome beginning 3.7mm anterior to the bregma. For each rat, nine consecutive sections are collected at an interval of 1mm and stored at -70°C. To visualize Evans blue staining, the slides are transferred to acetone at -20°C for 30 seconds and then rinsed in acetone at room temperature for 30 seconds.

The perfused microvessels (intraluminal Evans blue) in each section are inspected with fluorescent microscopy. Areas of perfusion deficits in the cortex and striatum are traced, calculated and expressed in mm^2.

Evaluation of BBB permeability

In the present study, BBB permeability was also measured following cerebral ischemia in order to determine the effects of hyperthermia on brain vascular health. BBB permeability was measured at 24 hours after MCA occlusion. BBB permeability was evaluated by fluorescent detection of extravagated Evans blue dye (Aoki et al., 2002).

At 24 hours after ischemic injury, animals are anesthetized again with 3.0% halothane and then maintained with 1.5% halothane in a 70:30 nitrous oxide:oxygen mixture oxide with a face mask. The surgical wound in the neck is reopened to isolate the right jugular vein. A PE-50 catheter is inserted into the right jugular vein as described in the section entitled "Detection of perfusion deficits". Evans blue (2% solution in saline, 0.2 mL/100g body weight) is injected into the right jugular vein and allowed to circulate for 15 minutes. Following that, animals are transcardially perfused with saline until colorless perfusion fluid is obtained from the right atrium. After decapitation, central and peripheral ischemic cortex regions are collected along with the striatum. Evans blue dye content is evaluated by a microplate fluorescence reader at an excitation wavelength of 620 nm and emission wavelength of 680 nm. The amount of extravagated Evans blue dye in each group is compared to normothermic sham operated rats and expressed as fold-change.

Hyperthermic fibrinolysis with tPA

Influence of hyperthermia on fibrinolysis with tPA was examined by incubating preformed clots at different temperatures in the presence or absence of tPA. The clots were formed as described in Chapter 2, and incubated in 2.5 mL of normal blood plasma with 2.5 mL of tPA reconstituted with saline and equivalent to 10 mg/kg. In the control groups, 2.5 mL saline was added instead of tPA. Progressive clot lysis was measured every hour and data were expressed as percentage of original length of the clots.

Experimental design

The present study consisted of three parts. In Part A, we examined whether hyperthermia increases perfusion deficits following ischemic injury. Due to premature mortality, 29 animals were used to obtain a total of 6 animals in each group for assessment of perfusion deficits. The animals were randomly assigned to the following groups: normothermic, sacrificed at 3 h after MCA occlusion (n=6), normothermic, sacrificed at 6 h (n=6), 39°C hyperthermia, sacrificed at 3 h (n=6), and 39°C hyperthermia, sacrificed at 6 h (n=6).

In Part B, we examined the effects of hyperthermia on BBB permeability following ischemic injury. Due to premature mortality, a total of 32 animals were used in this part of the study. The animals were randomly assigned to the following groups: normothermic sham operated (n=3), normothermic injured (n=6), 38°C hyperthermia sham operated (n=3), 38°C hyperthermia injured (n=6), 39°C hyperthermia sham operated (n=3), and 39°C hyperthermia injured (n=6).

In Part C, we examined the effects of hyperthermia on fibrinolytic activity of tPA using an *in vitro* study to determine whether hyperthermia affects ischemic damage by influencing enzymatic activity of tPA. Preformed clots were assigned to following groups: 37°C + saline (n=6), 37°C + tPA (n=6), 38°C + saline (n=6), 38°C + tPA (n=6), 39°C + saline (n=6), and 39°C + tPA (n=6).

Statistical analysis

The differences in perfusion deficits, BBB permeability, and fibrinolysis were analyzed with one-way ANOVA followed by the Holm-Sidak method. Differences were considered significant when $P<0.05$.

Results

Perfusion deficits

In the present study, we examined the mechanisms of the deleterious actions of hyperthermia in the ischemic brain injury. Previously, our lab has shown that perfusion deficits reduced over time after MCA occlusion (Wang et al., 2001b). To determine whether the deleterious effects of hyperthermia are due to delay in reduction of perfusion deficits, we compared the dynamic changes in perfusion deficits between normothermic and hyperthermic rats. Perfusion deficits were observed in the areas supplied by the MCA after the artery was occluded. The shape of the perfusion deficit was irregular, but the boundary between the perfused and non-perfused regions was usually very clear (Figure 4.1).

Figure 4.1: Perfusion deficits in the ischemic injured brain following embolization of a clot in the MCA

Figure 4.1: Perfusion deficits in the ischemic injured brain, induced by embolizing pre-formed clot into the MCA. The image is from a brain section of a 39°C hyperthermic rat sacrificed at 3 h after embolization.

Pooled data of perfusion deficits from the different groups are shown in Figure 4.2. In the normothermic rats, the perfusion deficits were 0.64±0.33 mm^2 (mean ± SEM) and 1.18±0.49 mm^2 at 3 h and 6 h after the MCA occlusion respectively. In the hyperthermic rats, the perfusion deficits were 4.01±0.75 mm^2 and 10.21±2.46 mm^2 at 3 h and 6 h respectively. Compared to the normothermic rats, perfusion deficits in the hyperthermic rats were significantly increased at both 3 h and 6 h ($P<0.05$).

Brain vascular permeability

Next we examined the effects of hyperthermia on brain vascular permeability. Temperature-induced exaggeration of BBB changes following global ischemia have been documented previously (Ginsberg and Busto 1998). We examined Evans blue dye extravasation in the brain following ischemic injury in normothermic and hyperthermic rats to determine the effects of hyperthermia on brain vascular permeability in focal ischemia. The amount of extravagated Evans blue dye in each group was compared to the normothermic sham operated rats and expressed as fold-change (Figure 4.3). Evans blue dye extravasation was significantly increased in both the peripheral ischemic cortex and striatum of the 39°C hyperthermic rats ($P<0.05$). There was no significant difference in dye extravasation among the sham operated groups.

Figure 4.2: Effects of hyperthermia on perfusion deficits following ischemic brain injury

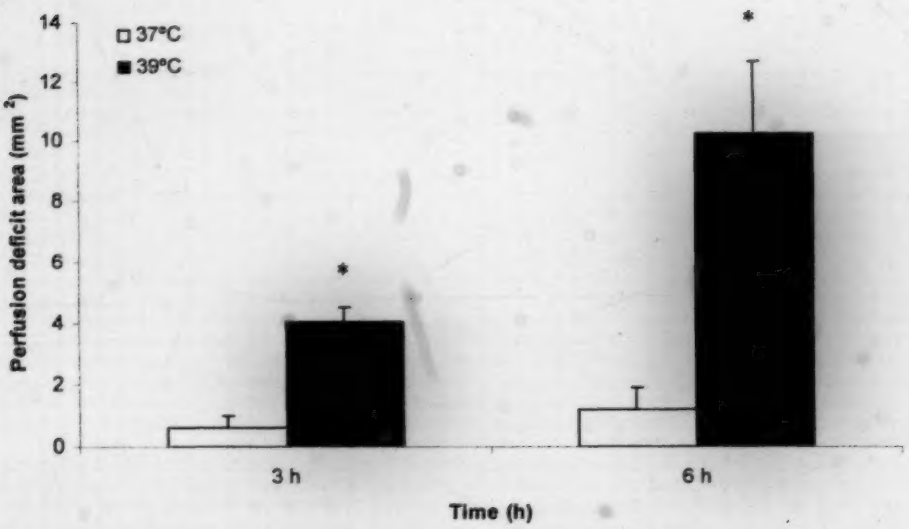

Figure 4.2: Effects of hyperthermia on perfusion deficits following focal cerebral ischemia. Animals were sacrificed at 3 h and 6 h after embolizing a pre-formed clot into the MCA. Each bar represents mean ± SEM (n=6). Hyperthermia (39°C) significantly increased perfusion deficits at both 3 h and 6 h following ischemic injury as compared to normothermic animals ($P<0.05$). * denotes significantly larger than in 37°C rats.

Figure 4.3: Effects of hyperthermia on BBB permeability following ischemic brain injury

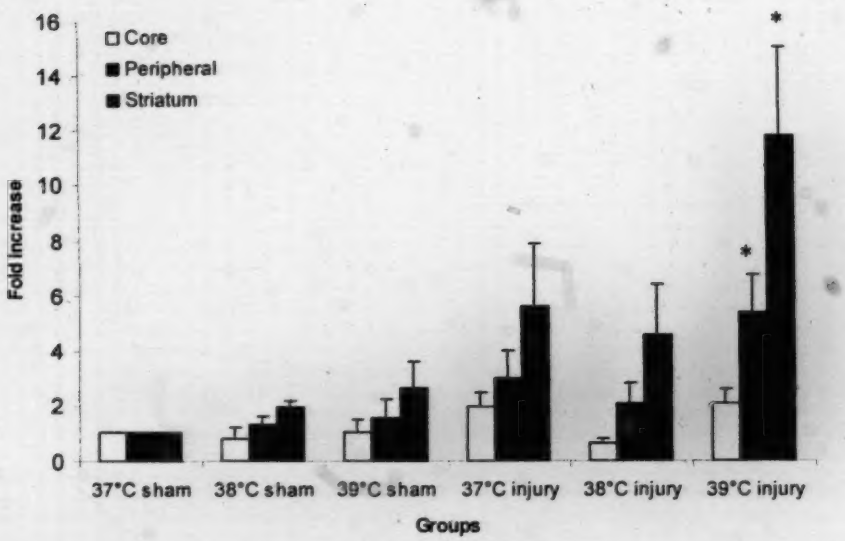

Figure 4.3: Effects of hyperthermia on BBB permeability following focal cerebral ischemia. Animals were sacrificed at 24 h after surgery, and extravasations of Evans blue were measured in the brain. Each bar represents mean ± SEM (n=6). Hyperthermia (39°C) significantly increased the levels of Evans blue in peripheral ischemic cortex and striatum following ischemic injury as compared to normothermic sham-operated animals ($P<0.05$). * denotes significantly larger than in 37°C sham-operated rats.

Hyperthermic fibrinolysis with tPA

In an *in vitro* study, the effect of hyperthermia on fibrinolysis with tPA was examined by incubating preformed clots at different temperatures in the presence or absence of tPA. In the 39°C + tPA group, the percent of original clot remaining was significantly smaller when compared to the 37°C + tPA group at 1 h, 2 h, and 3 h. In the 38°C + tPA group, the percent of original clot remaining was significantly smaller when compared to the 37°C + tPA group at 2 h and 3 h only (Table 4.1). There was no significant difference between clot sizes in the control groups at any of the three time points.

Table 4.1: Hyperthermic fibrinolysis with tPA [a]

	Time (h)		
	1	2	3
37°C + saline	94.1 ± 2.1%	88.2 ± 2.1%	79.8 ± 1.5%
37°C + tPA	81.1 ± 1.9%	64.9 ± 1.7%	48.6 ± 1.9%
38°C + saline	90.8 ± 2.5%	82.8 ± 1.6%	73.5 ± 2.9%
38°C + tPA	76.8 ± 2.2%	56.2 ± 3.0% *	29.5 ± 3.8% *
39°C + saline	90.2 ± 1.2%	84.8 ± 2.3%	72.4 ± 2.5%
39°C + tPA	72.8 ± 1.3% *	52.2 ± 2.2% *	18.1 ± 2.5% *†

[a] Preformed clots were incubated for 3 h and clot size was evaluated at 1 h, 2 h, and 3 h. Data are expressed as percent of original clot length remaining at each time point. Results are expressed as mean ± SEM (n = 6). * denotes significantly different compared to the 37°C + tPA group ($P<0.05$). † denotes significantly different compared to the 38°C + tPA group ($P<0.05$).

A number of mechanisms may contribute to the detrimental actions of hyperthermia on the ischemic brain injury. Brain temperature modulates ischemia-induced BBB opening with remarkable sensitivity. The extravasation of protein tracers across the barrier is markedly exaggerated by intraischemic hyperthermia of 39°C in a global model of ischemia (Ginsberg and Busto 1998). The results presented here show increased Evans blue dye extravasation following MCA occlusion in 39°C hyperthermic rats and thus confirm the findings that hyperthermia increases BBB permeability following ischemic injury. Since hyperthermia caused severe edema in the ischemic injured brain (discussed in Chapter 3), it is likely that the downstream microvasculature is compressed, thereby preventing restoration of blood flow, although the occlusion materials may be lysed either spontaneously or artificially with tPA. To test this hypothesis, dynamic changes in perfusion deficits between normothermic and hyperthermic rats were compared. Results indeed showed that hyperthermia significantly increased the perfusion deficits. These results show for the first time that hyperthermia contributes to the neurodestructive actions by delaying the re-establishment of perfusion in the ischemic injured brain.

Previously, *in vitro* studies have shown that hyperthermia facilitates tPA-mediated fibrinolysis. In one study fibrinolytic activity of tPA rose with increasing temperature as indicated by shorter lysis time and higher concentrations of D-dimer, a fibrin degradation product. After addition of tPA, the concentration of D-dimer was approximately tripled and time to complete clot lysis was approximately halved from 30°C to 40°C (Schwarzenberg et al., 1998). Our present results also show an increase in fibrinolytic activity of tPA at higher temperatures as indicated by a significant decrease in clot size at

higher temperatures. Combined with our results from Chapter 3, these results thus suggest that the increased fibrinolytic activity of tPA during hyperthermia is offset by its deleterious actions and the net effect of hyperthermia in ischemic brain injury is neurodestructive.

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Chapter 5: Endothelial function and stroke

So far, everything described above relates to the primary treatment of an acute stroke. Primary treatments for acute stroke require immediate attention, with therapies designed to restore blood flow as early as possible and to manage the associated complications. However, the scope of present studies will be shifted away from primary treatment to examine the role of endothelial dysfunction in development of atherosclerosis and stroke.

Cholesterol is a key link in the pathogenesis of atherosclerosis. An increase in LDL cholesterol results in a threefold increase in the risk of ischemic coronary artery disease (Castelli et al., 1992). The increased risk is particularly evident in individuals with familial hypercholesterolemia where most individuals with increased LDL cholesterol have symptomatic coronary artery disease (CAD) before the age of 30 years (Smilde et al., 2001; Nolting et al., 2003). The introduction of statins has resulted in significant decline in the risk of CAD in patients with increased LDL cholesterol (Werner et al., 2002). In contrast, an increase in HDL cholesterol is protective against atherosclerosis (Assmann and Nofer 2003; Lupattelli et al., 2003; Young et al., 2004). The major mechanism for the protective effect of increased HDL cholesterol relates to its ability to remove excess LDL cholesterol from atheromatus plaques and transport it to the liver for removal. Other mechanisms include the inhibition of adhesion molecules that facilitate the uptake of neutrophils and macrophages into the endothelium, inhibition of LDL oxidation in the arterial wall and an increase in endothelial NO production (Assmann and Nofer 2003; Young et al., 2004).

NO is an endothelium-derived signaling molecule that activates guanylate cyclase in vascular smooth muscle cells to induce relaxation. Diminished bioavailability of NO has a key role in early pathogenesis of hypercholesterolemia-induced vascular disease and atherosclerosis. Incubation of cultured ovine endothelial cells with 10μg/mL HDL resulted in a five-fold increase in eNOS activity (Yuhanna et al., 2001). HDL has also been shown to increase the abundance of eNOS protein in human vascular endothelial cells (Ramet et al., 2003).

Apoptosis of endothelial cells has been demonstrated in many cardiovascular diseases, including atherosclerosis. An intact monolayer of endothelial cells is required for the maintenance of normal vessel wall properties (O'Connell and Genest 2001). Endothelial cell apoptosis may lead to increased permeability of vascular endothelium and blood cell adhesion to the vessel wall, resulting in enhanced blood coagulation and thrombosis formation (Calabresi et al., 2003). HDL protects cultured human endothelial cells from TNF-α-induced apoptosis through inhibition of caspase-3 activity (Sugano et al., 2000). HDLs also suppress the mitochondrial pathway of apoptosis, which is induced by growth factor deprivation (Nofer et al., 2001).

Endothelial dysfunction is usually one of the earliest markers of atherosclerosis, and predisposes one to vasoconstriction and thrombosis (Verma and Anderson 2002). There are a number of ways endothelial dysfunction can be tested. Abnormalities in flow mediated brachial reactivity are associated with an increased risk of cardiovascular events, while coronary endothelial dysfunction predicts not only future cardiovascular,

but also cerebrovascular events, demonstrating the systemic nature of the atherosclerotic process (Perticone et al., 2001; Halcox et al., 2002; Targonski et al., 2003). More recently, it has been suggested that circulating EPCs may also be markers of endothelial function and cardiovascular risk. In healthy men, decreasing numbers of EPCs were strongly correlated with increased Framingham risk score, while a higher number of EPCs were correlated with improved endothelial function, as measured by flow-mediated brachial reactivity (Hill et al., 2003). EPC numbers are significantly decreased in subjects with elevated serum cholesterol, hypertension and diabetes (Hill et al., 2003) and smokers (Perticone et al., 2001), while EPC migratory ability is also impaired by hypertension (Vasa et al., 2001a).

Endothelial progenitor cells originate in bone marrow, and appear to be mobilized in response to vascular trauma or tissue ischemia (Takahashi et al., 1999), promoted in part by cytokine release and vascular endothelial growth factor (Asahara et al., 1999; Takahashi et al., 1999). eNOS is implicated in VEGF induced mobilization of EPCs through the activation of eNOS and matrix metalloproteinase-9 (MMP-9) pathways (Aicher et al., 2003). Following mobilization from the bone marrow, they subsequently migrate to the site of injury and differentiate into mature endothelial cells, promoting re-endothelialisation and neo-vascularisation (Murohara et al., 2000; Werner et al., 2003). This incorporation of EPCs has been clearly demonstrated at sites of induced carotid artery endothelial injury (Walter et al., 2002) as well as following cerebral (Hess et al., 2002; Zhang et al., 2002), myocardial (Kocher et al., 2001) and limb ischemia (Kalka et al., 2000a).

Effects of HDL cholesterol on this new marker of endothelial function have not been examined previously. In the present study, we examined the relationship between the levels of endothelial progenitor cells and serum HDL cholesterol levels in patients with cerebrovascular disease. Furthermore, using *in vitro* studies, we also examined how HDL cholesterol may increase the levels of EPCs by examining the effects of HDL treatment on cultured human endothelial progenitor cells. More specifically, we examined whether HDL treatment affected eNOS and MMP-9 levels in the EPCs, both of which are implicated in mobilization of EPCs from the bone marrow. In another study, we examined whether HDL treatment can inhibit homocysteine-induced activation of caspase-3 in EPCs, thereby preventing apoptosis and increasing the number of EPCs in the circulation.

Materials and Methods

This study was approved by the Research Ethics Review Board of the University of Alberta. Informed consent was obtained in writing from patients and control volunteers.

Patient population

The patients were recruited from the Stroke Prevention Clinic and the Acute Stroke Unit at the University of Alberta Hospital. Controls comprised patient family members and staff at the hospital.

Preparation of blood samples and cell culture

Peripheral blood (approximately 15 – 20mL) was collected from patients and volunteers at the Stroke Prevention Clinic at the University of Alberta Hospital. EPCs were cultured as described elsewhere (Hill et al., 2003). Briefly, mononuclear cells from peripheral blood were separated using Ficoll (Sigma) density gradient centrifugation. The cells were cultured in M199 medium supplemented with 20% fetal bovine serum, 100U/mL penicillin, and 100μg/mL streptomycin (Gibco).

Colony-forming units of endothelial progenitor cells

Initially $5x10^6$ mononuclear cells were seeded on 6-well human fibronectin-coated plates (Biocoat, Becton Dickinson). After 48 hours, $1x10^6$ nonadherent cells were re-seeded on 24-well fibronectin-coated plates to avoid contamination with mature endothelial cells. After seven days *in vitro*, endothelial colony-forming units were counted by two independent investigators. To confirm endothelial progenitor cell lineage, immunofluorescent cell staining was performed using CD34 (10μL; Becton Dickinson), CD31 (10μL; Serotec), and AC133 (10μL, Abjent) primary antibodies.

We divided the patients into three groups depending on the number of colony-forming units. This division was based on the criteria published by Hill et al., 2003. We measured a number of vascular risk factors and blood markers in the three categories and evaluated for any significant differences in the three groups (Table 5.1).

eNOS expression assay

EPCs isolated from healthy volunteers were cultured as described above. After seven days *in vitro*, the cultures were treated with HDL (Calbiochem) at concentrations of 10, 25, and 50μg/mL (n=15). After 24 h of incubation, the cells were harvested with Triton-based lysis buffer and the expression of eNOS was analyzed by an enzyme-linked immunosorbent assay for human eNOS protein (R&D Systems). Data are expressed as fold-change in eNOS protein expression in comparison to non-treated controls (n=15). Immunostaining with anti-eNOS monoclonal antibody (Biomol) and CD34 (Becton Dickinson) was also performed to visually verify the change in eNOS expression levels following HDL treatments. Horseradish conjugated anti-mouse secondary antibodies were purchased from Sigma.

Pro-MMP-9 expression assay

Pro-MMP-9 levels were assessed in EPCs isolated from healthy volunteers. After seven days *in vitro*, the cultures were treated with HDL at concentrations of 10, 25, and 50μg/mL (n=15). After 24 h of incubation, the cells were harvested using a cell lysis buffer and protein concentration was assessed. Pro-MMP-9 protein expression was assessed for each cell lysate (20μg of protein/sample) using an enzyme immunoassay for human MMP-9 (Daiichi). Data are expressed as fold-change in pro-MMP-9 levels compared to non-treated controls (n=15).

Caspase-3 activity assay

Caspase-3 activity was assessed in EPC cultures from healthy volunteers. The cultures were incubated with homocysteine (200μM), to induce apoptosis, and HDL at concentrations of 10, 25, and 50μg/mL for 24 h (n=15). After 24 h of incubation, the cells were harvested and caspase-3 activity was assayed using a Caspase-3 Colorimetric Assay Kit (BioVision). Briefly, the cells were removed from the wells by trypsinization followed by cell lysis and collection of supernatant to assay protein concentration. Caspase-3 activity was assayed for each cell lysate (20μg of protein/sample) using a Caspase-3 Colorimetric Assay Kit. Data are expressed as fold-change in Caspase-3 activity as compared to non-treated controls (n=15).

Statistical analysis

Results are expressed as means ± standard deviation (SD), median with range and number (percentages). The Mann-Whitney U test, analysis of variance (ANOVA), Kruskal-Wallis H test and the Pearson Chi-square test were performed to determine whether the number of vascular risk factors and blood markers were associated with three levels of EPC colony levels. Univariate linear regression was also used to relate age, gender, cigarette smoking, hypertension, diabetes, total cholesterol levels, fasting glucose, fasting homocysteine levels, creatinine levels, C-reactive protein, statin use and subject type (control, stable, and acute) to EPC colony levels. Variables with a ($P<0.20$) were entered into a multiple linear regression model to identify independent predictors of EPC colony levels. One-way ANOVA followed by the Holm-Sidak test was used to determine the differences between the groups in the *in vitro* studies. Results were

considered significant if the two-sided p-value was less than 0.05. The Statistical Package for Social Science SPSS 12.0 (released September 2003, standard version, copyright SPSS, 1989-2003) was used for data analysis.

Results

Blood HDL levels are directly correlated with EPC colony counts

Ninety-four subjects were included in the *in vivo* study. Sixty (63%) were male and thirty four (37%) were female. Mean age was 63.5 years (SD ± 12.86). Prevalence of cerebrovascular risk factors is given in Table 5.2. Seventeen percent of the subjects had no cerebrovascular risk factors, 27% of them had any one of the cerebrovascular risk factors, 30% had any two, 20% had any three and 6% had any four to five risk factors.

In our population the median EPC colony count was measured to be 7.55 [range 0-71]. We divided the patients into three groups based on the number of EPC colony counts {high count: median 19.75 [range 14-71]}, {intermediate count: median 7.5 [range 4.66-12.8]}, and {low count: median 2.4 [range 0-4.5]} (Table 5.1). The mean age of the patients in the three groups was 59, 63 and 68 years respectively. Increasing age significantly reduced the levels of EPC colonies (P=0.035). The mean total cholesterol, LDL cholesterol, triglycerides, fasting glucose, HbA1c, c-reactive proteins and creatine were not statistically different among the three groups. Similarly, the presence of smoking and hyperlipidemia were also not different. EPC colony count differed significantly (P=0.001) between acute [median 4.75; range 0-33], stable [median 7.28; range 0-43], and control subjects [median 15.53; range 4.3-71]. The median EPC colony

count in the control group was significantly different from acute (P<0.001) and stable groups (P=0.003) but there was no significant difference between the stable and acute groups (P=0.14).

Multiple linear regression analysis was performed to determine whether the number of EPC colonies was associated with age, gender, total cholesterol, LDL, HDL, fasting homocysteine, Framingham risk score, hypertension, diabetes, and type of subjects. This analysis showed that age (β= -0.209, P=0.027), HDL (β= 6.435, P=0.008) and the type of subject (acute vs control, β= -12.80, P=0.002; stable vs control β= -11.62, P= 0.001) were independent predictors of the number of EPC colonies (Table 5.3).

The HDL levels were directly related to EPC colony count after controlling for statin use (β = 8.642, P= 0.001). HDL cholesterol was significantly lower in individuals with low EPC colony levels (below 1.08 ± 0.26 mM,) compared with intermediate (1.28 ± 0.29 mM) and high HDL levels (1.57 ± 0.86 mM) (P=0.017) for those who were not on statin therapy (Table 5.4). An additional 27 patients were on statin therapy at the time of assessment and there appeared to be no difference in EPC colony counts in relationship to HDL levels in these individuals.

Next, we examined whether HDL has any direct effects on EPCs which could influence their health and numbers in circulation, thereby affecting the numbers of colony-forming units.

Table 5.1: Comparison of risk factors and blood markers to EPC colony levels

Factor	High Colony Count	Intermediate Colony Count	Low Colony Count	P-value‡
Age (in years)	59.72 ± 12.0	63.09 ± 15.38	68.06 ± 9.78	0.035
Total Cholesterol	5.38 ± 1.18	4.93 ± 1.24	4.84 ± 1.15	0.184
LDL	3.15 ± 1.07	3.04 ± 0.82	2.76 ± 0.94	0.290
Triglycerides	1.73 ± 1.03	1.73 ± 1.06	1.93 ± 1.48	0.758
Fasting Glucose	5.76 ± 1.82	5.98 ± 1.81	6.51 ± 2.73	0.426
HbA1c	0.057 ± 0.007	0.061 ± 0.012	0.061 ± 0.012	0.472
Fasting Homocysteine	9.66 ± 5.85	9.48 ± 4.19	12.26 ± 4.11	0.087
C-Reactive Protein	3.89 ± 3.69	4.87 ± 4.76	4.72 ± 6.76	0.819
Framingham Risk Score	9.28 ± 8.4	11.92 ± 7.62	14.88 ± 9.61	0.034
Creatinine	115.11 ± 66.87	90.63 ± 24.75	93.56 ± 42.24	0.245
Hypertension	16 (50)	14 (45.2)	23 (74.2)	0.047†
Diabetes	5 (15.6)	5 (16.1)	12 (38.7)	0.049†
Hyperlipidemia	17 (53)	14 (45.2)	16 (51.6)	0.799†
Smoker	8 (25)	11 (35.5)	7 (23.3)	0.514†
Type of subject				
Acute	2 (6.3)	7 (22.6)	9 (29)	
Stable	18 (56.3)	20 (64.5)	20 (64.5)	0.008
Control	12 (37.5)	4 (12.9)	2 (6.5)	
Total subjects per group	32	31	31	

Results are expressed as mean ± standard deviation for continuous variables and Number (percentage) for categorical variables. ‡P-value has been calculated through ANOVA and Kruskal-Wallis test whenever appropriate. †P-value has been calculated through Pearson Chi-square test.

Table 5.2: Cerebrovascular risk factors in the study population (n = 94)

Cerebrovascular Risk Factors	Number of patients (%)
Hypertension	53 (55.8)
Hyperlipidemia	48 (50.5)
Smoking	26 (27.4)
Diabetes Mellitus	22 (23.2)
History of MI	10 (10.5)
Peripheral vascular disease	7 (7.4)
History of hyperhomocysteinemia	6 (6.3)

Table 5.3: Independent predictors of levels of EPC colonies from multiple linear regression analysis

Factors	Coefficients (β)	Standard error(SE) of β	P-value
Age in years	-0.209	0.093	0.027
HDL	6.435	2.381	0.008
Subject type:			
Stable vs. control	-11.62	3.235	0.001
Acute vs. control	-12.80	4.058	0.002

Table 5.4: Effect of HDL on EPC colony forming units after adjusting for statin therapy

		EPC colony forming units (level)			P-value‡
	Statin	High	Intermediate	Low	
HDL(mM)	Yes	1.102 ± 0.36	1.08 ± 0.35	1.14 ± 0.28	0.925
	No	1.57 ± 0.86	1.28 ± 0.29	1.08 ± 0.26	0.017

‡ P-value has been calculated through the non-parametric Kruskal-Wallis test.

HDL increases eNOS protein expression in EPCs

NO, an important endothelial-derived signaling molecule, acts on the vascular smooth muscle cells to induce relaxation of the vessel wall. A decrease in NO availability plays a key role in early pathogenesis of atherosclerosis. HDL has been previously shown to increase eNOS activity (Yuhanna et al., 2001) and protein abundance (Ramet et al., 2003) in cultured endothelial cells. Here, we examined whether HDL has any effects on eNOS expression in primary human endothelial progenitor cells isolated from blood of healthy volunteers. Our results show that HDL treatment significantly increases eNOS protein expression in EPCs at 10, 25, and 50μg/mL concentration levels ($P<0.001$). HDL treatment for 24h increased eNOS protein expression to 1.71 ± 0.48-fold (mean ± SD), 1.82 ± 0.45-fold, and 3.00 ± 0.66-fold of control, at concentrations of 10, 25, and 50μg/mL respectively. eNOS protein fold-change with 50μg/mL was also significantly higher as compared to 10 and 25μg/mL HDL ($P<0.001$) (Figure 5.1). Immunostaining for eNOS using anti-eNOS monoclonal antibody also showed an increase in eNOS expression following HDL treatments (Figure 5.2).

Figure 5.1: eNOS protein expression fold-change following HDL treatments

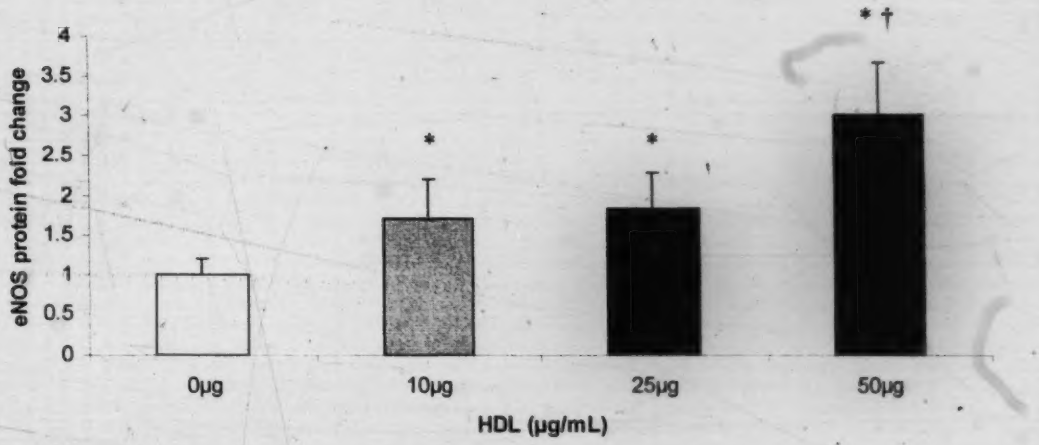

Figure 5.1: eNOS protein expression fold-change following HDL treatments. EPCs were incubated in normal medium or medium supplemented with HDL at concentrations of 10, 25, and 50μg/mL for 24 h. HDL treatment significantly increased eNOS protein expression in EPCs at 10, 25, and 50μg/mL concentrations ($P<0.001$). Each bar represents mean ± SD. * denotes significantly larger than control group. † denotes significantly larger than 10 and 25μg/mL HDL groups.

Figure 5.2: eNOS immunostainging using anti-eNOS monoclonal antibody

Figure 5.2: eNOS immunostainging using anti-eNOS monoclonal antibody. Immunostaining for eNOS protein expression in EPCs showed an increase in eNOS fluorescence following treatment with (B) 50μg/mL HDL as compared to (A) controls.

HDL decreases pro-MMP-9 levels in EPCs

VEGF-induced progenitor cell mobilization from the bone marrow is dependent on local levels of MMP-9 (Asahara et al., 1999; Heissig et al., 2002), which is activated by NO-mediated S-nitrosylation (Gu et al., 2002). Pro-MMP-9 is significantly decreased in bone marrow plasma of $Nos3^{-/-}$ mice. These mice also showed a profoundly reduced basal activity of pro-MMP-9 and also a blunted increase in pro-MMP-9 activity in response to 5-fluorouracil treatment (Aicher et al., 2003). HDL has been shown to inhibit oxidized-LDL induced increase in MMP-9 protein expression (Xu et al., 1999). Here, we examined whether HDL has any effects on EPC mobilization by increasing pro-MMP-9 levels in EPCs secondary to HDL-mediated increase in eNOS levels. Our results show that HDL significantly decreases pro-MMP-9 levels in EPCs at a concentration of 50μg/mL (P=0.002) but not at 10 (P=0.08) or 25μg/mL (P=0.09). HDL treatment decreased pro-MMP-9 levels to 0.79 ± 0.26-fold (mean ± SD) in the 10μg/mL group, 0.84 ± 0.26-fold in the 25μg/mL group, and to 0.62 ± 0.30-fold in the 50μg/mL group, as compared to controls. There was no significant difference between the 10μg/mL, 25μg/mL, and 50μg/mL groups (P>0.05) (Figure 5.3).

Figure 5.3: Pro-MMP-9 level fold-change following HDL treatments

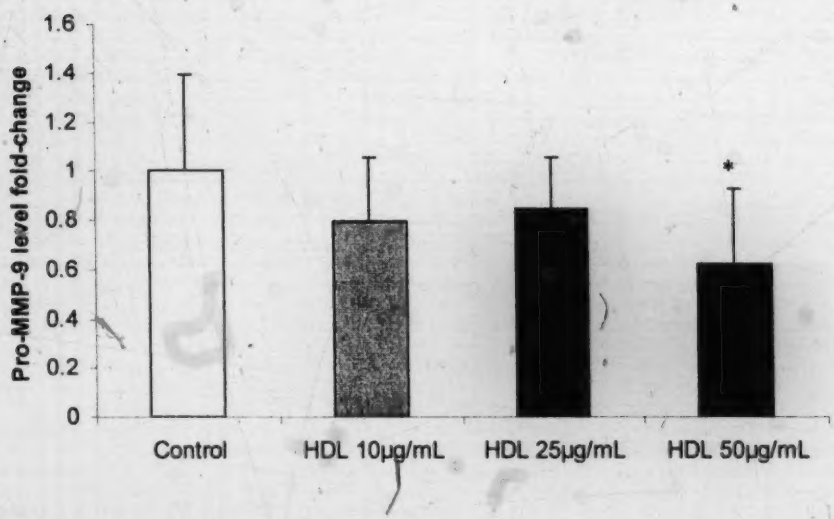

Figure 5.3: Pro-MMP-9 level fold-change following HDL treatments. EPCs were incubated in normal medium or medium supplemented with HDL at concentrations of 10, 25, and 50µg/mL for 24 h. HDL treatment significantly decreased pro-MMP-9 levels in EPCs at the concentration of 50µg/mL (P=0.002) but not at 10 (P=0.08) and 25µg/mL (P=0.09). Each bar represents mean ± SD. * denotes significantly smaller than control group.

HDL inhibits caspase-3 activation in EPCs

Integrity of the endothelium is very important for the maintenance of normal vessel wall properties. An intact endothelium is essential to mask the thrombogenic molecules that lie underneath the monolayer of endothelial cells. Endothelial cell apoptosis may lead to increased permeability of vascular endothelium, resulting in enhanced blood coagulation and thrombosis formation (Calabresi et al., 2003). HDL exerts a protective effect by interfering with the apoptotic stimuli to which endothelial cells are exposed. HDL protects cultured human endothelial cells from TNF-α-induced apoptosis through inhibition of caspase-3 activity (Sugano et al., 2000). Here we examined whether HDL treatment can inhibit homocysteine-induced activation of caspase-3 in EPCs isolated from blood of healthy volunteers. Our results show that homocysteine treatment significantly increased caspase-3 activity in the EPCs to 3.21 ± 1.39-fold (mean ± SD) as compared to control ($P<0.001$). Following HDL treatment, caspase-3 activity was decreased to 2.07 ± 0.76-fold, 2.12 ± 0.97-fold, and 1.65 ± 1.10-fold of control, in the 10, 25, and 50μg/mL HDL groups respectively. HDL treatment at 10 ($P=0.004$), 25 ($P=0.006$) and 50μg/mL ($P<0.001$), significantly decreased caspase-3 activity as compared to the homocysteine-treated group. However, caspase-3 activity in the 10 and 25μg/mL HDL groups was still significantly higher as compared to the control group. There was no significant difference between control and 50μg/mL HDL groups ($P=0.09$) or between any of the other treatment groups when compared to each other (Figure 5.4).

Figure 5.4: Caspase-3 level fold-change following HDL treatments

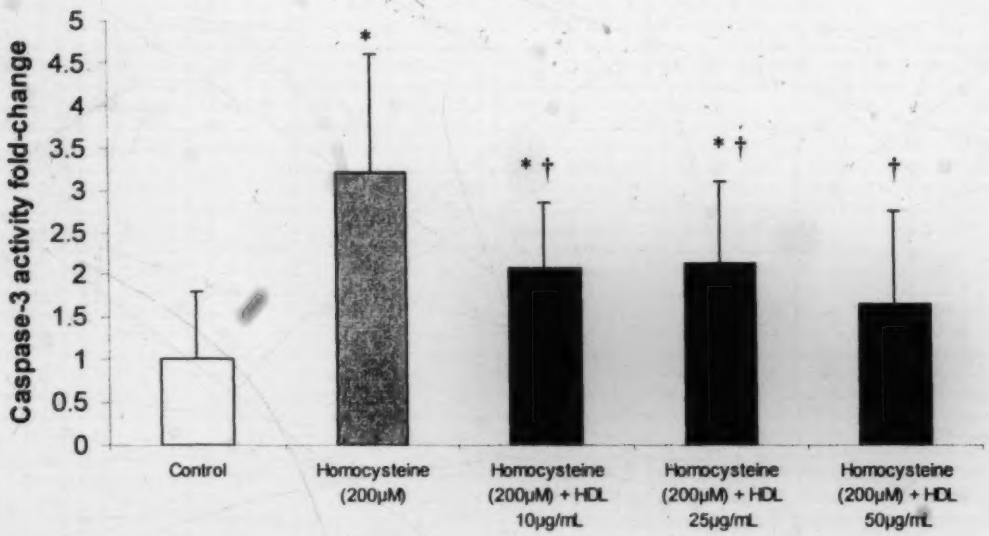

Figure 4. Caspase-3 activity change following homocysteine and HDL treatments. EPCs were incubated with homocysteine (200μM) and HDL at concentrations of 10, 25, and 50μg/mL for 24 h. Homocysteine treatment significantly increased caspase-3 activity as compared to control ($P<0.001$). HDL, at concentrations of 10 ($P=0.004$), 25 ($P=0.006$), and 50μg/mL ($P<0.001$), significantly decreased caspase-3 activity as compared to the homocysteine-only treated group. Caspase-3 activity in the 10 and 25μg/mL HDL group was still significantly higher as compared to the control group. Each bar represents mean ± SD ($n = 15$). * denotes significantly larger than control group. † denotes significantly smaller than homocysteine (200μM) group.

In summary, the present study shows that there is a positive relationship between blood levels of HDL cholesterol and levels of circulating EPC colony-forming units. As shown by our *in vitro* study, the relationship between the two may be related to the ability of HDL to increase eNOS expression in EPCs and, more importantly, increase their numbers by prevention of EPC apoptosis through inhibition of caspase-3 activity.

*Sections of this chapter have been submitted to Atherosclerosis journal for publication.

Chapter 6: General discussion

The work described in this thesis comprised two different but related aspects of the neurological phenomenon of stroke. The first section of the work described involved working with an animal model of stroke, more specifically examining the effects of hyperthermia (increased body temperature) on progression of ischemic injury in a focal embolic model of stroke in rats. A rat model of stroke was chosen for these studies because cerebral ischemia in this model most closely resembles the clinical situations, and when compared to gerbils, cats, and dogs, the rat cranial circulation is most similar to that of the human (Yamori et al., 1976). A reliable model of embolic stroke in rats had already been established in our lab and employed in investigating various mechanisms of cerebral ischemia and the efficacy of a variety of therapeutic agents (Wang et al., 2001c) and, therefore, there was no need to establish another newer model, although further improvements to the model were made in the course of this study in order to increase the reliability, reproducibility, and efficiency of the procedure. The model was also modified to incorporate protocols for hyperthermia treatment, as described in Chapter 2.

Once the stroke model was properly instituted, the scope of the study was furthered into examining the effects of hyperthermia on infarction volume following embolic occlusion of the MCA. The results showed that treatment with controlled hyperthermia significantly increased neuronal damage following ischemic injury in an embolic model of stroke in rats. Treatment with hyperthermia (brain temperature of 39°C) during and for 3 h following embolic occlusion of the MCA significantly increased infarct volume

and neurological deficits. Moreover, hyperthermia also resulted in increased mortality following embolic stroke.

Results from this initial experiment paved the way for further experiments using this model in order to evaluate the effects of hyperthermia on the efficacy of thrombolytic treatment with tPA during stroke. Since tPA is the only approved thrombolytic therapy for acute stroke (Albers 1999; Traynelis and Lipton, 2001), it was important to examine the effects of a common clinical complication, such as hyperthermia, on its efficacy when administered in a setting of acute embolic stroke. In order to assess these effects, brain infarction volume was measured in normothermic and hyperthermic rats after they were treated with tPA following embolic occlusion of MCA. Results from this part of the study confirmed our previous findings that hyperthermia significantly worsened the outcome following cerebral ischemia in an embolic model of stroke in rats.

Moreover, our data also showed that hyperthermia masks the neuroprotective effects of tPA, as treatment with tPA significantly reduced infarction volume in normothermic rats whereas in moderately hyperthermic rats (38°C) the neuroprotective effects were less pronounced, and no improvement was seen in severely hyperthermic rats (39°C). Another important parameter for treatment efficacy is improvement in functional recovery. However, our results showed that tPA treatment also did not improve functional recovery, measured with behavioral tests, in the hyperthermic rats. In addition, tPA treatment also failed to decrease mortality in the hyperthermic rats. These

data thus clearly show that thrombolytic therapy with tPA is not effective in ischemic brain injury in the presence of hyperthermia.

Following this, we went on the examine some possible mechanisms through which hyperthermia may influence the extent of cerebral ischemic injury. One of the common phenomena seen in the brains of our hyperthermic ischemic animals was tissue edema. Although brain edema was only significantly increased in the 38°C rats, the trend in 39°C rats was also towards increased edema. Because of this increased edema in the ischemic injured brain, we hypothesized that it is likely that the downstream microvasculature may be compressed, thereby preventing restoration of blood flow, although the occlusion materials may be lysed either spontaneously or artificially with tPA. To test this hypothesis, we examined the dynamic changes in perfusion deficits between normothermic and hyperthermic rats. Results indeed showed that hyperthermia significantly increased the perfusion deficits following ischemic injury. The results presented here showed for the first time that hyperthermia contributes to the neurodestructive actions by delaying the re-establishment of perfusion in the ischemic injured brain.

Since brain temperature also modulates ischemia-induced BBB opening with remarkable sensitivity, we further hypothesized that additional mechanisms may also contribute to the detrimental actions of hyperthermia on the ischemic brain injury. Experiments in global ischemia models had shown that the extravasation of protein tracers across the barrier is markedly exaggerated by intraischemic hyperthermia of 39°C (Ginsberg and

Busto 1998). Therefore we also examined the effects of moderate and severe hyperthermia in our embolic model of focal cerebral ischemia. Our results showed increased Evans blue dye extravasation following MCA occlusion in 39°C hyperthermic rats and thus confirm the findings that hyperthermia increases BBB permeability following ischemic injury. tPA is known to potentiate NMDA-receptor-mediated signaling and neuronal death. tPA enhances NMDA-evoked Ca^{2+} influx, which has been shown to be one of the critical events in excitotoxic neuronal cell death (Nicole et al., 2001). Thus, an increase in the passage of tPA through the BBB during hyperthermia may explain why hyperthermia masks the beneficial effects of tPA-induced thrombolysis.

Next, we went on to examine the effects of hyperthermia on fibrinolytic activity of tPA in an *in vitro* study. Previous *in vitro* studies have shown that hyperthermia facilitates tPA-mediated fibrinolysis and we wanted to confirm that we are also producing similar effects and not inactivating the enzyme and abolishing its fibrinolytic activity. In one study fibrinolytic activity of tPA rose with increasing temperature as indicated by shorter lysis time and higher concentrations of D-dimer, a fibrin degradation product. After addition of tPA, the concentration of D-dimer was approximately tripled and time to complete clot lysis was approximately halved from 30°C to 40°C (Schwarzenberg et al., 1998). Our results showed an increase in fibrinolytic activity of tPA at higher temperatures as indicated by significant decrease in clot size at higher temperatures. These results thus suggest that the increased fibrinolytic activity of tPA during hyperthermia is offset by its deleterious actions and a net effect of hyperthermia in ischemic brain injury is neurodestructive.

In summary, the experiments from the first section of this work show that hyperthermia significantly increases infarction volume following ischemic insult in an embolic model of cerebral ischemia. Hyperthermia also reduces the neuroprotective effects of tPA in ischemic brain injury. Moreover, hyperthermia increases BBB permeability and causes a delay in the re-establishment of brain perfusion, which in turn accelerates the progression of ischemic penumbral region to irreversibly damaged tissue. The delay of reperfusion is likely due to increased edema during hyperthermia. These findings are clinically relevant as the data clearly suggest that hyperthermia should be combated assiduously where thrombolytic therapy with tPA is used for treatment of ischemic stroke patients.

After successful completion and publication of the above-mentioned experiments, the focus shifted to secondary stroke prevention, the second aspect of stroke discussed in this compilation. More specifically, we examined the effects of HDL cholesterol on a new emerging marker of endothelial dysfunction, namely EPCs. This required shifting the focus of the work from animal studies to the clinical setting and working with human samples. Experiments in this section comprised both clinical and *in vitro* studies. Details of these experiments have been presented previously in Chapter 5, and, therefore, only the results will be discussed here.

Endothelial dysfunction is one of the earliest events in the pathogenesis of atherosclerosis (Ferro 2004). Recent studies have shown that low levels of HDL cholesterol are associated with endothelial dysfunction. This endothelial dysfunction can be corrected when the HDL cholesterol levels are increased with medical therapy (Kuvin et al., 2002;

Aicher et al., 2003). In a study of 184 patients with vascular disease where brachial artery flow-mediated vasodilatation was studied with low and high HDL levels, low levels were associated with poor relaxation in response to acetylcholine. The authors postulated that the low HDL modulated endothelial function through a lack of LDL cholesterol oxidation inhibition and a concomitant over-expression of adhesion molecules (Lupattelli et al., 2003). In another study of 151 patients where brachial artery flow-mediated dilation was studied, a highly significant correlation was evident with decreasing levels of HDL cholesterol and flow mediated endothelial dysfunction. Similar findings were also reported by Lupatelli et al. in a study of 107 patients (Lupattelli et al., 2002). In an interesting report by Spieker et al., patients with low HDL cholesterol were treated with reconstituted HDL (80 mg/kg) and brachial artery function monitored to a challenge with acetylcholine. There was a significant improvement in brachial reactivity as the levels of HDL cholesterol increased (Spieker et al., 2002), suggesting the direct positive effects of HDL cholesterol on endothelial function. Finally, in a similar study of 28 patients with low or normal HDL cholesterol, Kuvin et al. reported that treatment with niacin for three months resulted in an improvement in brachial artery flow-mediated relaxation (Kuvin et al., 2002).

Results from our clinical study showed a significant relationship between low levels of HDL cholesterol and the resultant low levels of EPC colony counts. This effect was independent of the age of the patients, other vascular disease risk factors and the presence or absence of symptomatic vascular disease. We did not find a similar effect between

EPC colony levels and LDL cholesterol levels, triglycerides or an increase in C-reactive proteins.

We then went on examine some possible mechanisms through which HDL may be improving EPC levels, and results from our *in vitro* studies confirm the positive relationship between HDL cholesterol and the endothelial progenitor cells. Our results show that HDL increases eNOS expression in EPCs. eNOS, a heme-containing enzyme, is constitutively expressed by vascular endothelial cells and generates relatively low levels of NO at physiological conditions. NO induces relaxation in the vascular smooth muscle cells and serves as an atheroprotective factor (Aicher et al., 2003; Assmann and Nofer 2003; Iwakura et al., 2003). NO also mediates a variety of physiological functions, including neovascularization. eNOS-deficient mice are characterized by an impaired ischemia-induced neovascularization (Murohara et al., 1998). Recent studies support the concept that eNOS-derived NO exerts additional functions by regulating stem or progenitor cell mobilization from the bone marrow (Aicher et al., 2003; Iwakura et al., 2003). eNOS becomes activated in response to multiple stimuli, including hemodynamic shear stress and agonists of diverse G protein-coupled cell-surface receptors. Incubation of cultured endothelial cells with HDL activates eNOS through the scavenger receptor-BI (Yuhanna et al., 2001). HDL also enhances eNOS expression in cultured human endothelial cells (Kuvin et al., 2002; Ramet et al., 2003). *In vivo* studies provide additional support to the concept that HDL prevents endothelial dysfunction by promoting endothelial NO production. Niacin treatment in patients with low levels of

HDL causes an elevation of plasma HDL with parallel increase of NO-mediated vasodilation (Kuvin et al., 2002).

HDL-mediated increases in levels of eNOS in EPCs could augment their numbers in the peripheral circulation through increased mobilization from the bone marrow. MMP-9 is essential for VEGF-induced mobilization of bone marrow-derived progenitor cells (Hiessig et al., 2002). eNOS-deficient mice show significantly reduced levels of pro-MMP-9 in the bone marrow plasma (Aicher et al., 2003). MMP-9 is activated by VEGF and NO (Gu et al., 2002; Hiratsuka et al., 2002). The local increase of MMP-9 by the hematopoietic and stromal compartments of the bone marrow results in the cleavage of membrane bound Kit ligand and, therefore, the release of soluble Kit ligand. Release of soluble Kit ligand enables the transfer of endothelial and hematopoietic progenitor cells from the quiescent state to a proliferative state. Once the progenitor cells proliferate, they enter the blood stream and enhance neurovascularization by incorporation into vessels at sites of ischemic injury (Heissig et al., 2002). However, there are also reports that MMP-9 is inactivated by NO (Gurjar et al., 1999). Our results show that although eNOS levels are increased in EPCs following HDL treatment, pro-MMP-9 levels were found to be decreased in the 50μg/mL HDL-treated group. This suggests that positive effects of HDL on EPCs do not involve MMP-9-mediated mobilization of these progenitor cells from the bone marrow. Other studies have shown that granulocyte-colony stimulating factor (G-CSF), a cytokine typically used for mobilization of $CD34^+$ cells in patients, releases the proteinases elastase and cathepsin G from neutrophils and appears not to rely on MMP-9 for stem cell mobilization. Elastase and cathepsin G cleave the adhesive

bonds on stromal cells that interact with integrins on hematopoietic stem cells (Levesque et al., 2001). A decrease in pro-MMP-9 produced by HDL is also consistent with the finding that HDL inhibits the oxidized-LDL induced increase in MMP-9 protein in human monocyte-derived macrophages (Xu et al., 1999).

The positive effects of HDL on circulating EPCs may involve an elevation in their numbers through an increase in survival period and prevention of apoptosis. In our clinical study, low levels of HDL were associated with low levels of EPC colony-forming units, suggesting that HDL may be involved in maintenance of circulating pools of EPCs and their colony-forming units. Indeed, results from our *in vitro* study confirm that HDL is involved in maintenance of circulating pools of EPCs by preventing their apoptosis through inhibition of caspase-3 activity. Endothelial cell apoptosis may lead to enhanced blood coagulation by exposing the blood to underlying thrombogenic molecules. Therefore, a continuous and intact monolayer of endothelial cells is important for maintenance of normal vessel wall properties. Incorporation of EPCs at sites of ischemic injury can rescue endothelial function by repairing damaged vessels or by forming new vessels at sites of injury (Hess et al., 2002; Zhang et al., 2002). Findings from other studies support our data since HDL has been shown to protect cultured human endothelial cells from TNF-α induced apoptosis in a dose-dependent manner at physiological concentrations of HDL, and this effect is mediated through inhibition of caspase-3 activity (Sugano et al., 2000).

There are exciting data on the effects of conventional vascular risk factors on circulating EPC levels. There is an inverse relationship with increasing levels of risk factors and the number of EPCs. In a study by Vasa *et al.*, smoking appeared to be the most important inhibitor of EPC levels and hypertension slowed migration of EPCs (Vasa et al., 2001). In a recent study by Hill *et al.*, it was shown that increasing Framingham risk factors inhibit EPC levels. The decrease in levels of EPC colony-forming units very significantly correlated with abnormalities in vascular reactivity to ischemia. The authors suggested that abnormalities in EPC colony levels may be an important biological surrogate marker of vascular function (Hill et al., 2003). Our study showed that there is a positive relationship between blood levels of HDL cholesterol and levels of circulating EPC colony-forming units. As shown by our *in vitro* study, the relationship between the two may be related to the ability of HDL to increase eNOS expression in EPCs and, more importantly, to increase their numbers by prevention of EPC apoptosis through inhibition of caspase-3 activity.

Concluding remarks

In summary, the work presented here started out with examining the effects of hyperthermia on therapeutic implications in acute stroke in animals. The results established that hyperthermia masks the beneficial effect of tPA when administered in the setting of focal embolic stroke and therefore stresses the importance of assiduously combating fever in stroke patients when therapy with tPA is to be used. In the second leg of the work presented here, the focus was shifted from an acute stroke setting to stroke prevention in relation to endothelial health. The work showed that there is a positive

relationship between HDL cholesterol and levels of circulating EPCs and that HDL may regulate EPC levels by increasing eNOS expression and prevention of EPC apoptosis.

Chapter 7: References

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